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GRANT NUMBER DAMD17-94-J-4366

TITLE: Nature and Prognostic Significance of Alterations in the
DCC and E-Cadherin Genes in Breast Cancer

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REPORT DATE: January 1999

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
January 19993. REPORT TYPE AND DATES COVERED
Final (15 Jul 94 - 14 Jan 99)

4. TITLE AND SUBTITLE

Nature and Prognostic Significance of Alterations in the DCC and E-Cadherin Genes in Breast Cancer

5. FUNDING NUMBERS

DAMD17-94-J-4366

6. AUTHOR(S)

David L. Rimm, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Yale University School of Medicine
New Haven, Connecticut 06520-80478. PERFORMING ORGANIZATION
REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-501210. SPONSORING / MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

19990330 132

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Breast cancer, like other epithelial tumors, is a highly complex and multi-factorial process. Oncogenes involved in metastasis represent a important area of exploration toward understanding the progression of this disease. This grant focuses on the role of Deleted in Colon Cancer (DCC) and E-cadherin. Ample evidence has been published implicating both in breast cancer. In the last year, we have concentrated on the regulation of expression of these molecules in breast cancer and the regulation of adhesion. Specifically, we have shown the E-cadherin promoter appears to be regulated by trans-activation, as opposed to methylation, and further localized regions of the promoter that are critical for expression. We have also shown the expression of E-cadherin protein is concordant with expression of α -catenin and β -catenin, but not with p120ctn. Its expression is frequently altered, but rarely lost in breast ductal carcinoma, and not significantly associated with outcome. We have also examined expression of Met, the HGF/SF receptor in ductal carcinoma, since it is thought to be responsible for down-regulation of adhesion. We found overexpression of Met is a highly significant, independent predictor of decreased survival in the 20-25% of cases were it is expressed, even in node negative breast cancer.

14. SUBJECT TERMS
breast cancer

15. NUMBER OF PAGES

164

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT

Unclassified

18. SECURITY CLASSIFICATION
OF THIS PAGE

Unclassified

19. SECURITY CLASSIFICATION
OF ABSTRACT

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

FOREWORD

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PI Signature

2/12/99

Date

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Introduction:

General Background:

Breast cancer, like other epithelial tumors, is a highly complex and multi-factorial process. The biological events that occur and the causes are wide ranging. Among these events, genetic alterations, both somatic and inherited, are likely to play a major role. This premise has led to a broad search of both specific genes and chromosomal regions in efforts to correlate genetic changes to tumor behavior with the goal of improving diagnostic and therapeutic tools.

An extensive progress report on this grant was submitted about one and half years ago (September 1997). This progress report represents an update on that report. Specific negative results outlined in that report will not be repeated here, nor will summaries of previous publications. For each point in the Statement of Work, there will be an update on progress since the last report in September of '97 and reference to manuscripts included in the appendix.

Background of previous work:

Human cancers are generally thought to arise through a multi-stage evolutionary process driven by inherited and somatic mutations of genes and clonal selection of variant progeny with progressively increasing tendency toward aggressive, unregulated growth. Progression occurs largely through somatic mutation of oncogenes and tumor suppressor genes, although some mutations in these same genes are inherited, making a given individual more susceptible to developing malignancy.

The range of oncogenes, tumor suppressor genes and chromosomal locations implicated in breast cancer is broad and ever expanding. These will not be discussed here, rather we will concentrate on the DCC and E-cadherin genes and their chromosomal locations, the focus of this grant.

Both DCC and E-cadherin have been implicated as tumor suppressor genes. The phenotype of a tumor suppressor gene is tumor progression as a result of alteration or loss of expression of the normal protein encoded by these genes. Often the phenotype is expressed as a result of loss of one of the two alleles present in normal cells. This allelic loss has been termed loss of heterozygosity (LOH). LOH has been postulated to inactivate the tumor suppressor gene located in the affected chromosomal region (Knudson's Hypothesis)(Knudson,1993). A region on chromosome 18q (18q21-23) has been observed to show LOH in 35-70% of breast cancers(Thompson *et al.*,1993). This region contains the DCC gene. In colorectal cancers, DCC represents a classic tumor suppressor gene, showing LOH in over 70% of cases and grossly detectable somatic gene rearrangements in 15% of the cases (Cho and Fearon,1995).

E-cadherin maps to human chromosome 16q21.1 (Berx *et al.*,1995b), a region that has also been examined for LOH. Between 30 and 50% of breast cancer cases have shown LOH on 16q(Sato *et al.*,1991). Initially this was thought to correlate with decreased protein expression as multiple publications that showed decreased expression of E-cadherin in breast cancer(Gamallo *et al.*,1993; Moll *et al.*,1993; Oka *et al.*,1993; Rimm *et al.*,1995b; Siitonen *et al.*,1996), but more recently, only lobular carcinoma shows true E-cadherin mutations (Berx *et al.*,1995a). The significance of LOH at 16q remains to be shown. It is possible other tumor suppressor genes lie within that region.

Functionally, E-cadherin is well characterized. A hypothetical cartoon of the cadherin-based transmembrane adhesion complex (figure 1) shows the components of the complex and the

associated cortical cytoskeleton. The Greek letters, α , β and γ indicate the corresponding catenins. Plakoglobin has been shown to be identical to γ -catenin. Although we have evidence that α -catenin can bind both spectrin (Lombardo *et al.*, 1994) and actin (Rimm *et al.*, 1995) and that it exists as a dimer (Koslov *et al.*, 1997), the linkages shown are hypothetical. The interactions between β - and α -catenin are shown using β -catenin as an example of a member of the *arm* family that binds directly to both E-cadherin and α -catenin. Other than plakoglobin, it is not yet known if other members of the *arm* family can participate in this interaction. The stoichiometry of the interactions of β -catenins and plakoglobin are unknown although there is evidence for a single *arm* family member per cadherin (Hinck *et al.*, 1994). The *src* substrate, p120CAS (now called p120ctn), binds at a different site than the other members of the *arm* family (Shibamoto *et al.*, 1995) and cannot bind to α -catenin (Daniel and Reynolds, 1995). Protein tyrosine phosphatase μ is shown to indicate a direct connection to E-cadherin in some cells (Brady-Kalnay *et al.*, 1995) however other PTPases have also been implicated in the complex interacting with β -catenin (Balsamo *et al.*, 1996; Fuchs *et al.*, 1996). Not shown are many tyrosine kinases, including growth factor receptors and *src* family tyrosine kinases.

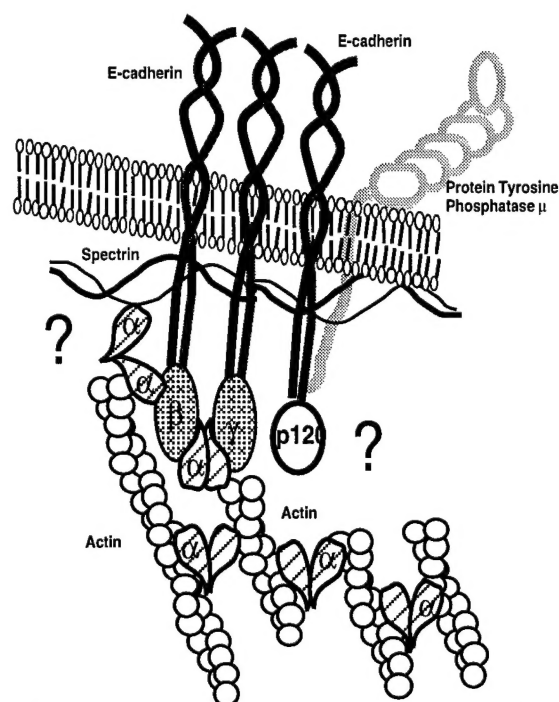


Figure 1 A schematic cartoon of the cadherin-based adhesive junction showing the proteins that interact with E-cadherin on the cytoplasmic face and their potential connections to the cytoskeleton.

Subject and Scope of Present Work:

In the original application, three technical objectives were proposed with the goal of understanding the role of the adhesion molecules DCC and E-cadherin in breast cancer. The initial task focused on use of LOH to assist in confirming that these adhesion molecules are important in the pathogenesis of breast cancer. Ultimately, the long term goals were to apply the findings of these studies to improving the diagnosis and management of breast cancer.

The following specific aims/tasks were proposed in effort to meet the above goals:

- 1) To determine the frequency of LOH affecting chromosomes 16q and 18q in primary breast cancers; to identify the common region affected by LOH on each of these chromosomes; and to identify the possible associations of such LOH events with clinical and histopathological features.
- 2) To identify specific genetic alterations in the DCC and E-cadherin genes in breast cancers
- 3) To address the functional role of the DCC and E-cadherin genes as tumor suppressor genes in breast cancer

Evolution of original aims due to progress in the field:

The original aims addressed above were largely the direction of this effort. As there has been substantial progress in the field on many aspects of this work, as described both above and below, there has been some modifications in our focus. Primarily, less emphasis has been placed on Task 1, as copious evidence has developed that both DCC and E-cadherin are important in the pathogenesis of breast cancer. As the ultimate goal is to improve diagnosis and management of breast cancer, we have expanded our effort on two fronts. We have increased our efforts on the proteins associated to the cytoplasmic domain of E-cadherin, with emphasis on understanding both their function and regulation. This includes the catenins, and has also expanded to include growth factor receptors that have been associated with down regulation of adhesion, specifically Met, the hepatocyte growth factor/scatter factor receptor. We have also increased our efforts on understanding the regulation of expression of DCC and E-cadherin. As a result of our own work, as well as many others in the field, it is evident that mutation in these genes may represent an important, but less prominent mechanism of loss of function than alterations in the regulatory process. As a result, the bulk of the effort in this grant focused on regulation of expression.

Body:

SOW task 1: To determine the frequency of loss of heterozygosity (LOH) affecting chromosomes 16q and 18q in primary breast cancers; to identify the common region affected by LOH on these chromosomes; and to identify the possible associations of such LOH events with clinical and histopathological features.

As described in our Sept, '97 progress report, this section has been discontinued for reasons described above in the section entitled "evolution of original aims due to progress in the field".

SOW task 2a: Studies of DCC and E-cadherin gene and protein expression in breast cancer cell lines

This section was completed in Year 1. This work is described in Pierceall et al, 1995 in *Oncogene*. The DCC aspect of this task is also addressed a manuscript by Meyerhardt et al. Both of these reprints were included in the September, '97 progress report.

In light of changes in the studies outlined in the "evolution" section above, task 2a has been extended, with extensive focus on the mechanisms of E-cadherin expression. This work describes efforts to understand the E-cadherin gene expression by characterization of 5' flanking sequences and insertion of these sequences into a reporter gene (luciferase) system. Contrary to previous published work, (Graff *et al.*,1995) we find that modulation of expression is a function of trans-acting factors as opposed to changes in methylation of the promoter sequence. This work is described in detail in a paper by Ji et al (Ji *et al.*,1997) included in the appendix.

SOW task 2b: Studies of DCC, E-cadherin and α - and β -catenin in primary tumors

Expression studies were undertaken primarily by immunohistochemistry as examinations based on mRNA were unnecessary. Immunohistochemical studies have focused on E-cadherin and cadherin associated proteins including α - and β -catenin and p120ctn. This work has culminated in a manuscript that was published in the *American Journal of Pathology*. A reprint is included in the appendix. This represents an extension of the original aims as discussed in the "evolution" section above.

Studies of DCC on primary tumors, although desirable, have been hampered by lack of sufficient protein levels in either tumor or normal tissue for productive immunohistochemical studies. No further progress has been made toward this aspect of the task.

Given the difficulties with DCC antibodies, this area of investigation was expanded over the last year to include examination of the *c-met* oncogene. It was shown by Shibamoto (Shibamoto *et al.*,1994) and others (Barth and Nelson, personal communication) that the *c-met* gene product, Met, is associated with phosphorylation of β -catenin and decreased cell-cell adhesion. Met, the receptor for HGF/SF (hepatocyte growth factor/scatter factor), is a tyrosine kinase type growth factor receptor whose stimulation results in a range of cellular changes beyond decreased cell-cell adhesion including motility and mitogenesis (Komada and Kitamura,1993) and tubulogenesis (Sachs *et al.*,1996).

In the Sept. 97 we described work on examination of Met expression in breast cancer. We found that it is an independent predictor of survival with predictive value equivalent to that associated with lymph node status. We conclude that expression of Met in invasive ductal carcinoma of the breast is a strong, independent predictor of decreased survival and may be a useful prognostic marker to identify a subset of lymph node negative patients with more

aggressive disease. This work is detailed in a manuscript by Ghoussoub et al. that has been published in the journal CANCER and is included in the appendix.

This work has been extended by further study of 113 node-negative breast cancer cases. This new cohort is non-overlapping with the previous study, but arrives at similar conclusions. Specifically, even in node negative patients, Met expression has independent predictive value with respect to prognosis. Furthermore, in this study we found that there is a correlation between Met expression and nuclear and histologic grade. Notably, the group that is strongly positive for Met and also has nuclear grade of 3 has only a 60% 5 yr. Survival, compared to a 97% survival for patients negative for Met and with nuclear grade 1 or 2. This work is shown in detail and discussed in the appended submitted manuscript by Camp et al. that has been submitted to the journal CANCER.

SOW task 2c.1: Studies to identify and characterize specific mutations in E-cadherin genes

Specific efforts to find mutations in E-cadherin in human breast tumors has met with limited success elsewhere in the field. Although lobular carcinoma of the breast has been found to have some gene mutations by three groups (Kanai, et al.,1994; Berx, et al.,1995a; Candidus, et al.,1996), no mutations in E-cadherin have yet been published for any cases of ductal carcinoma.

We have used the more recent work of Berx and colleagues (Berox *et al.*,1996) as a guide for synthesis of PCR primers and detection mutations by SSCP. We have also shifted our focus to only lobular carcinomas, as the Berx group and others have never found mutations in ductal carcinoma, while the frequency of E-cadherin mutations in lobular carcinoma is reported as high as 50%. Our revised goal was to examine lobular carcinomas in efforts to correlate mutations in E-cadherin with outcomes, including recurrence and survival. Our progress on this effort was described in the Sept. '97 report. Although we were able to use PCR and SSCP as a mechanism to examine the E-cadherin gene for mutations, we found no mutations in the 20 lobular carcinoma cases examined and hence this area of investigation was abandoned.

As discussed above in the evolution of aims section, new data has suggested that regulation of adhesion may occur also through mutations in the catenins (Morin *et al.*,1997; Rubinfeld *et al.*,1997). Both of our labs have moved in the direction of evaluating β -catenin exon 3 sequence. It has been found that mutations in exon 3 in a critical region for ser/thr phosphorylation can play a critical role in oncogenesis (Peifer,1997). These studies, although not specifically on breast tissue, address a pathway (the *wnt* pathway) that has been implicated in breast cancer and were, in part, funded by this grant. Three manuscripts are either in press or submitted related to these issues and they are included in the appendix.

SOW task 2c.2: Alterations in DCC expression and gene structure in breast cancers

This task was addressed in the Sept '97 report and has not been further pursued.

SOW task 3a: Transfection, isolation and preliminary characterization of breast cancer lines with E-cadherin, β - and α -catenin and DCC cDNAs.

Efforts toward this task are well underway but have met with limited success. We have produced cDNAs encoding all 4 proteins and successfully cloned them into a range of eukaryotic expression vectors. We have focused predominantly on the CMV driven systems and either lipofection or electro-poration as mechanisms for transfection. To date we have been unable to produce and stable transformants of α -catenin or E-cadherin in any breast cancer cell

lines. We have had limited success in a non-breast cancer line (Clone A) and are using this system to optimize conditions(Roe *et al.*,1996). Unfortunately, the transformation of these genes seems to confer a significant growth rate disadvantage, and although we are able to see transient expression and even stable transfection by immunofluorescence at early time points, minimal passaging of the cells to produce sufficient numbers to analyze, or even freeze, have resulted in loss of expression. The observation that α -catenin overexpression results in a dramatically decreased growth rates has been made by another lab as well and was recently published(Bullions *et al.*,1997).

To circumvent this problem, we have been construction of expression vectors and cell lines for use in the tetracycline inducible system described by Gossen and colleagues(Gossen et al,1992; Gossen *et al.*,1995). We have made good progress toward this aim, in that we have produced stable Tet-ON MDA-MB-468 breast cancer cells. We are now in the process of making various α -catenin and β -catenin constructs and doing transfections with these new clones.

Similar difficulties have been encountered with DCC transfection. Work on DCC was described in the Sept. 97 report and no further progress was made toward this task.

SOW task 3a: Further characterization of the *in vitro* growth properties of breast cancer lines transformed with E-cadherin, β - and α -catenin and DCC cDNAs.

Although this task is targeted for years 2-4, it was dependent on the success of task 3a, which has not yet occurred.

Conclusions:

- I.** Expression of E-cadherin, α -catenin and β -catenin in breast cancer cell lines is altered in a large number of cases.

Alterations are often, but not always, seen in one member of the complex where the others are expressed normally.

Alterations occur at both the protein and message levels in breast cancer cell lines.

Mutations are seen β -catenin which affect its subcellular localization and may affect its role in adhesion.

- II.** Using a luciferase reporter gene, the E-cadherin promoter shows transcriptional activity in 3 breast cancer lines that express E-cadherin protein as well as 3 lines that do not.

Expression of the reporter gene in an unmethylated construct parallels that of the endogenous E-cad gene in each cell line, suggesting methylation does not down regulate the E-cad promoter. Furthermore, treatment with 5-aza-2'deoxyctidine does not reactivate E-cad in lines where E-cad is not transcribed. We conclude the down regulation of transcription at the E-cad promoter is not a function of methylation but rather a trans-acting factor.

- III.** Unlike breast cancer cell lines, expression patterns showed high concordance for E-cadherin, α -catenin and β -catenin, but p120ctn is independently regulated in essentially all human breast cancer cases.

Altered patterns of expression are present in 80% of invasive ductal carcinomas of the breast for the three concordant antigens, but somewhat less for p120ctn. Only p120ctn shows complete loss of expression, and then only in about 10% of the cases.

High levels of concordance of expression between all three proteins and rare examples of complete loss of expression, along with the fact that 36% of cases with normal α -catenin expression are node positive (have metastasized) suggests that down regulation of the adhesion molecules (at either the transcriptional or translational level) must play a more significant role than somatic mutation in affecting loss of adhesive function.

- IV.** Expression of Met, the HGF/SF receptor is lost in 75-80% of breast cancers.

The 20-25% of cases that show strong expression of Met have significantly worse outcomes than those that lose expression

Even in node-negative breast cancer patients, Met expression is a strong predictor of poor outcome (Relative risk for death = 5 increasing to 33 when combined with high nuclear grade).

Expression of Met has independent predictive value for poor prognosis as assessed by the Cox Model, with a magnitude approximately equivalent to, but independent of that seen for lymph node metastasis.

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14. Camp, RL, Rimm, EB and Rimm, DL (1999) *Met Expression is Associated with Poor Outcome in Node-Negative Breast Cancer* Cancer, submitted
15. Schofield, K, D'Aquila, TG, and Rimm, DL (1999) *E-cadherin expression is a sensitive and specific method for detection of carcinoma cells in fluid specimens* Cancer Cytopathology submitted

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Dillon, D., D'Aquila T., Fearon E.R., and Rimm, D.L. (1996) *Altered Expression Of α -Catenin In Breast Cancer*. Mod. Pathol. 9(1):16a

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Personnel with Salary Support:

David L. Rimm, P.I. (Yale University School of Medicine)	10%
Eric R. Fearon, Co-P.I. (Univeristy of Michigan Medical School)	10%
Tom D'Aquila, Technician (Yale University)	100%
Xiaodong Ji, Research Assoc. II (Univ. of Michigan)	100%

Appendix:

Appended are all manuscripts funded by this grant, with the exception of those sent in the previous progress report of September 1997. They are:

1. Schofield, K., D'Aquila, T., and Rimm, D.L. (1997) The cell adhesion molecule, E-cadherin, distinguishes mesothelial cells from carcinoma in fluids. *Cancer Cytopath* 81(5):293-298
2. Gold, J., Bao, L., Ghoussoub, R.A.D., Zetter, B.R., and Rimm, D.L. (1997) *Localization and Quantitation of Expression of the Cell Motility related protein Thymosin β 15 in Human Breast Tissue* *Modern Pathology* 10(11):1106-1112
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11. Camp, RL, Rimm, EB and Rimm, DL (1999) *Met Expression is Associated with Poor Outcome in Node-Negative Breast Cancer* *Cancer*, submitted
12. Schofield, K, D'Aquila, TG, and Rimm, DL (1999) *E-cadherin expression is a sensitive and specific method for detection of carcinoma cells in fluid specimens* *Cancer Cytopathology* submitted

The Cell Adhesion Molecule, E-Cadherin, Distinguishes Mesothelial Cells from Carcinoma Cells in Fluids

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Presented as a poster at the American Society of Cytopathology Meeting, Denver, Colorado, November 5-9, 1996.

Supported by grants from the William and Catherine Weldon Donaghue Foundation for Medical Research and the U.S. Army (DAMD17-94-J4366).

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Received May 29, 1997; accepted June 26, 1997.

BACKGROUND. The distinction between benign reactive mesothelial cells and well differentiated carcinoma can be difficult in pleural, peritoneal, and especially pericardial fluids. E-cadherin is an adhesion protein that is specifically expressed in cells of epithelial lineage. In this study, anti-E-cadherin antibodies were used to identify and distinguish carcinoma cells from reactive mesothelial cells.

METHODS. Pleural, peritoneal, and pericardial fluids were prepared using the Cytoc Thin Prep[®] processor. The specimens were comprised of a mix of 45 cases that were diagnosed as carcinoma, suspicious, or reactive by Papanicolaou staining of routine material seen by the authors' service. Routine immunologic techniques were used with a commercially available E-cadherin antibody.

RESULTS. In most cases of carcinoma, tumor cells showed a strong positive membranous reaction product (32 of 37). This included four cases that were not cytologically diagnosed as malignant, but subsequently proved to be malignant. E-cadherin staining was not observed in five tumors, two of which were not expected to express this protein. One benign case showed cells staining for E-cadherin, although the cells were not malignant by morphologic criteria. Because this case was a surgical pelvic washing, these cells more likely were epithelial contaminants than true false-positives.

CONCLUSIONS. The epithelial specific cell-cell adhesion marker E-cadherin reliably distinguishes reactive mesothelial cells from carcinoma and is a useful adjunctive test to distinguish benign reactive mesothelial cells from well differentiated carcinoma cells in fluid specimens. *Cancer (Cancer Cytopathol)* 1997;81:293-8.

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KEYWORDS: adhesion, cadherin, effusion, cytopathology, Thin Prep[®].

Although cytologic distinction between metastatic carcinoma and reactive mesothelial cells in pleural, peritoneal, and pericardial fluids can be very difficult, the staging of tumors and subsequent treatment of patients depends on it. One reason for the difficulty is that mesothelial cells can take on a wide range of so-called "atypical" and/or "reactive" changes in response to many different forms of stimuli, including infection, cirrhosis, and pneumonia. The goal of this study was to find a marker that could reliably and reproducibly distinguish reactive mesothelial cells from neoplastic cells.

One method for distinguishing between carcinoma and benign mesothelial cells is immunostaining. Typically, a panel including antibodies to keratin, CD15 (LeuM1), carcinoembryonic antigen (CEA), and occasionally BerEP4 or B72.3 is used. Nearly all carcinomas are positive for keratin, but so are most mesothelial cells, both reactive and malignant. CEA is more helpful because nearly all mesothelial

cells are negative for CEA. The main weakness of this marker is that only 50–67% of the adenocarcinomas express it.^{1,2} CD15 (LeuM1) also is helpful because many carcinomas stain with this marker but mesothelial cells do not.³ Similarly, both BerEP4⁴ and B72.3⁵ have been shown to selectively recognize adenocarcinomas. Unfortunately, these methods are not much better than the simple, old-fashioned method of periodic acid–Schiff diastase staining, which reveals approximately 50% of the adenocarcinomas, and when done properly, is negative in mesothelial cells. As a result, cocktails of multiple markers have been tried and even logistic regression analysis has been used to select the best panel.⁶ To the authors' knowledge, to date, there is no single marker in common usage that definitively separates cells of epithelial lineage from those of mesenchymal lineage.

E-cadherin, an epithelial specific homotypic adhesion protein, has the potential to be a marker with high sensitivity and specificity for the detection of carcinoma cells. It is a 120-kilodalton transmembrane glycoprotein whose calcium sensitive homotypic adhesion is the primary stabilizing interaction in cell-cell adhesion and a signal for polarization and cell differentiation.⁷ It has been shown that interruption of the function of any of the components of the cadherin-based transmembrane complex leads to loss of epithelial cell-cell adhesion.⁸ Cells of mesenchymal origin, like mesothelial cells, express a related cadherin called N-cadherin, and do not express E-cadherin.^{9,10} E-cadherin was used in this study to mark tumors of epithelial lineage. Although E-cadherin is lost in some tumors (e.g., lobular carcinoma of the breast¹¹ and gastric signet ring cell carcinoma¹²), nearly all well differentiated epithelial tumors maintain expression of this marker. Antibodies to the extracellular domain of E-cadherin do not cross react with N-cadherin. In this study, the authors tested the hypothesis that well differentiated carcinoma can be distinguished from mesothelial cells by antigenic recognition of E-cadherin expression.

METHODS

Forty-five specimens were selected from the study service between May 1995 and April 1996. The cases included 13 pleural fluids, 28 peritoneal/pelvic washings, and 4 pericardial fluids. The 45 cases were selected at random from the routine cytologic material seen by the study service over the last year. The cases were chosen to test the new reagent on a broad range of specimens and were not representative of the average distribution of cases received during that period. The cytologic diagnoses used were those assigned at "sign-out" by the attending physician on service. They

included cytologic diagnoses of carcinoma, suspicious, atypical, and reactive by Papanicolaou (Pap) stain. Each case had surgical follow-up or a previous specimen in the surgical pathology files. All cases were obtained from the Yale Pathology Department's Critical Technologies Cytology Collection with permission from the Yale Human Investigation Committee, protocol #8219. The cases are summarized in Table 1.

All pleural, pericardial, and pelvic/peritoneal fluids were submitted for routine cytologic examination in the usual manner. Specimens were spun down and the supernatant fluid was discarded. The pellet was then resuspended in CytoLyt[™] (Cytoc Corp., Boxborough, MA) and centrifuged again. The supernatant fluid was decanted again and 2 drops of the pellet were placed in PreservCyt[™] (Cytoc Corp., Boxborough, MA) solution. After 15 minutes, the specimen was processed using the Cytoc Thin Prep[™] (Cytoc Corp., Boxborough, MA) processor. Finally, the specimens were stained using the Pap staining technique for routine screening by the cytotechnologist and sign-out by the pathologist.

To evaluate E-cadherin expression, at least two Thin-Prep slides were made from each case. One was treated with E-cadherin antibody and the other was used as a negative control. Thin-Prep slides were rinsed for 5 minutes in tap water followed by 5 minutes in Tris-buffered saline (TBS) (150 mM NaCl and 20 mM Tris [pH = 8]). Slides then were blocked for 20 minutes with diluted normal serum from the Vectastain ABC-AP Kit (Vector Laboratories, Burlingame, CA). After blocking, slides were washed once in 1X TBS. E-cadherin monoclonal antibody (Transduction Laboratories, Lexington, KY) was diluted 1:250 in 1X TBS, 3–4 drops applied per slide, and then incubated for 30 minutes. The slides then were washed again in 1X TBS for 5 minutes. Then 3–4 drops of diluted biotinylated antibody from the Vectastain ABC-AP kit were applied per slide and incubated for 30 minutes, followed by a 5-minute wash in 1X TBS. Vectastain ABC-AP Reagent was added (3–4 drops) and the slides were incubated for an additional 30 minutes followed by a 5-minute wash in 1X TBS. Vector Red alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA) was applied (3–4 drops) and the slides were incubated for 10 minutes. The slides then were washed for 5 minutes in tap water, counterstained with hematoxylin, and coverslipped.

Stained slides were scored by the cytotechnologist and the pathologist. All the cases were scored independently using a binary system: present (positive) or absent (negative). There were no discrepancies in scoring between the pathologist and the cytotechnologist. Chi-

TABLE 1
Summary of Cases in Current Study

Case no.	Site	Cyto diagnosis	E-cad	Surgical
1	Peritoneal	Adenocarcinoma	(+)	Adenocarcinoma
2	Peritoneal	Suspicious	(+)	Adenocarcinoma
3	Pleural	Adenocarcinoma	(+)	Adenocarcinoma
4	Pleural	Adenocarcinoma	(+)	Adenocarcinoma
5	Peritoneal	Adenocarcinoma	(+)	Adenocarcinoma
6	Pleural	Adenocarcinoma	(+)	Adenocarcinoma
7	Pericardial	Nonsmall cell carcinoma	(+)	Adenocarcinoma
8	Peritoneal	Negative	(-)	Negative
9	Pleural	Adenocarcinoma-large cell	(-)	Adenocarcinoma-large cell
10	Pericardial	Adenocarcinoma	(+)	Adenocarcinoma
11	Pericardial	Negative	(-)	Negative
12	Pericardial	Negative	(-)	Negative
13	Peritoneal	Adenocarcinoma	(+)	History of adenocarcinoma
14	Peritoneal	Adenocarcinoma	(+)	Adenocarcinoma
15	Peritoneal	Adenocarcinoma	(+)	Adenocarcinoma
16	Pleural	Adenocarcinoma	(+)	History of adenocarcinoma
17	Pleural	Negative	(+)	Adenocarcinoma
18	Pleural	Melanoma	(-)	Melanoma
19	Pleural	Negative	(-)	Negative
20	Peritoneal	Adenocarcinoma	(+)	Adenocarcinoma
21	Peritoneal	Negative	(-)	Negative
22	Pleural	Adenocarcinoma	(+)	Adenocarcinoma
23	Pleural	Negative	(-)	Negative
24	Peritoneal	Adenocarcinoma	(+)	History of adenocarcinoma
25	Pleural	Adenocarcinoma-signet ring	(+)	Adenocarcinoma-signet ring
26	Peritoneal	Adenocarcinoma	(+)	Adenocarcinoma
27	Pleural	Adenocarcinoma	(+)	Adenocarcinoma
28	Peritoneal	Adenocarcinoma	(+)	Adenocarcinoma
29	Peritoneal	Negative	(+)	Negative
30	Peritoneal	Suspicious	(-)	Adenocarcinoma
31	Pleural	Atypical	(+)	Adenocarcinoma
32	Peritoneal	Dysgerminoma	(+)	Dysgerminoma
33	Pelvic wash	Adenocarcinoma	(+)	Adenocarcinoma
34	Pelvic wash	Adenocarcinoma	(+)	Adenocarcinoma
35	Pelvic wash	Negative	(+)	Adenocarcinoma
36	Pelvic wash	Adenocarcinoma	(+)	Adenocarcinoma
37	Pelvic wash	Adenocarcinoma	(+)	Adenocarcinoma
38	Pelvic wash	Adenocarcinoma	(+)	Adenocarcinoma
39	Pelvic wash	Adenocarcinoma	(+)	Adenocarcinoma
40	Pelvic wash	Adenocarcinoma	(-)	Adenocarcinoma
41	Pelvic wash	Negative	(-)	Negative
42	Pelvic wash	Adenocarcinoma	(+)	Adenocarcinoma
43	Pelvic wash	Hepatocellular Ca	(-)	Hepatocellular Ca
44	Peritoneal	Adenocarcinoma	(+)	Adenocarcinoma
45	Peritoneal	Adenocarcinoma	(+)	Adenocarcinoma

Cyto: cytologic; E-cad: E-cadherin; Ca: carcinoma; +: positive; -: negative.

square statistics were performed using the StatView 4.5 program for the Apple Macintosh computer.

RESULTS

Cells treated with E-cadherin antibody are shown in Figures 1 and 2. The staining pattern was predominantly membranous, but in many cases also was cytoplasmic. In benign cells the majority of the cadherin was at the

membrane, with the minority in biosynthetic pathways in the endoplasmic reticulum or Golgi's complex (not shown). In tumor cells the distribution was more heterogeneous. Both benign and malignant epithelial cells stained but mesothelial cells, lymphocytes, and inflammatory cells did not. In each case, accompanying Pap stained cells and negative controls counterstained with hematoxylin showed the specificity of the antibody

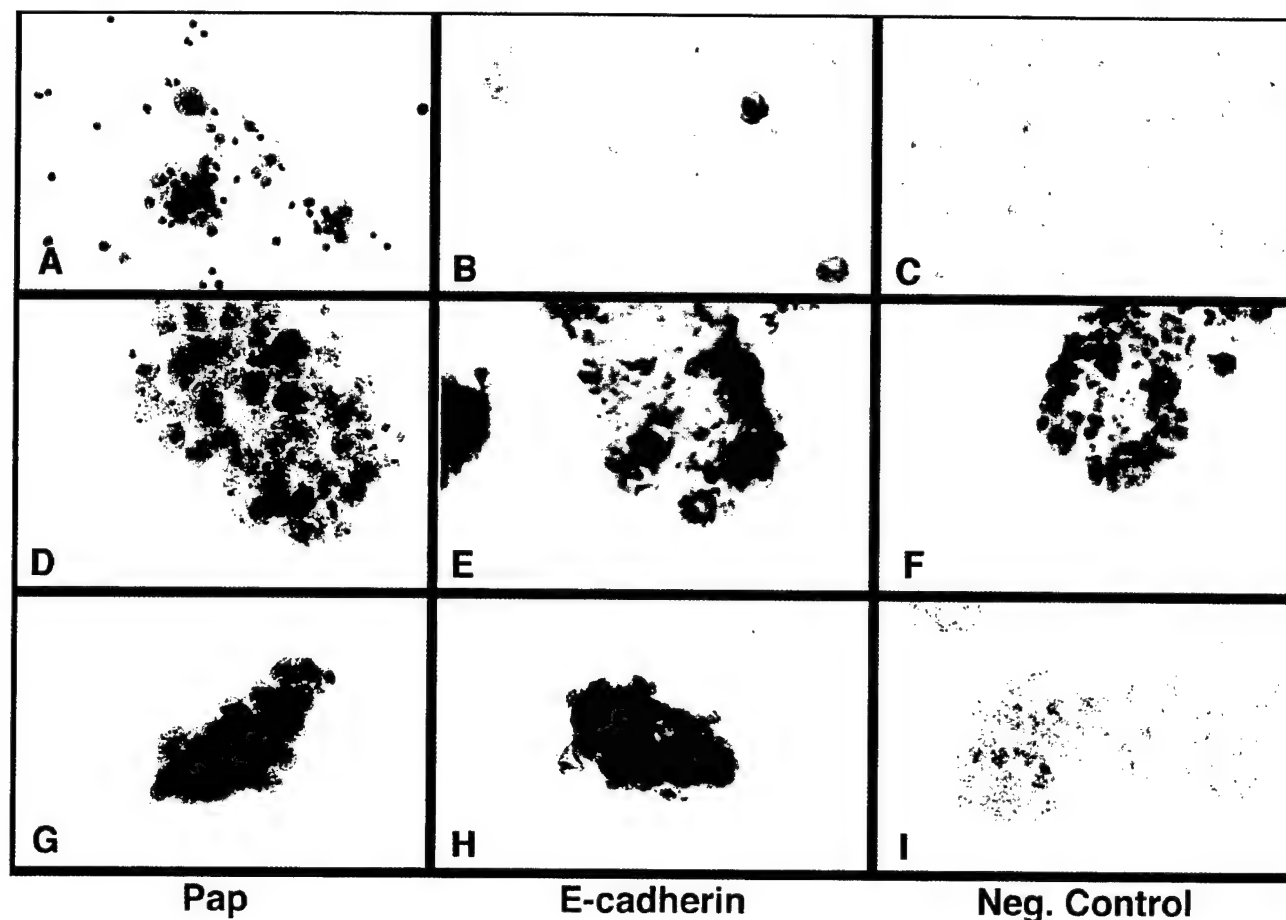


FIGURE 1. Examples of E-cadherin staining at low power (A–C: $\times 200$) and high power (D–I: $\times 600$). The left column (A, D, and G) shows Papanicolaou (Pap) stained images. Equivalent areas from a second Thin Prep are shown in the middle column after E-cadherin staining and detection with Vector RedTM chromogen (Vector Laboratories, Burlingame, CA) (B, E, and H). The column on the far right shows a negative control (neg control) for each case (C, F, and I).

staining. Figure 1 shows three classic example cases including one with single malignant cells (Fig. 1A–C) and others in clusters at higher power. Figure 2 shows an example of a “difficult” case that was determined to be atypical after the Pap stain. Suspicious clusters, as observed in the lower Pap stained frame, were rare and not well preserved. A duplicate Thin PrepTM overlaid with anti-E-cadherin antibody showed rare (two to three per slide) groups of unambiguous positively stained clusters, including a membranous pattern (Fig. 2B). Some atypical-appearing cells did not stain, which suggests that they were not epithelial (Fig. 2E), but rather reactive mesothelial cells.

The results are summarized in Table 1. E-cadherin staining was not subtle, and easily scored as either positive or negative, with no disagreements between the cyto-technologist and the pathologist. The summary of E-cadherin scoring of all the cases is shown in Table 2, split

by cytologic diagnosis. Although most cases (28 of 32) that were malignant had E-cadherin staining, 4 cases did not. Thirteen cases were not determined to be malignant by cytology. Of these, three cases that were termed “suspicious” showed malignant cells on a subsequent surgical procedure. E-cadherin staining was positive in two of these three cases. Of the ten cytologically negative cases, three showed E-cadherin staining. Two of these three cases were found to be positive for ovarian adenocarcinoma on a subsequent surgical specimen, suggesting they were not false-positive cases. On reexamination of the third case, a pelvic wash specimen from a patient with an ovarian abscess, the cells that stained did not appear to be morphologically malignant and may have been squamous cells or other cells of epithelial origin that artifactually entered the wound during the surgical procedures. The final tabulation of staining, split by final obtainable diagnosis, is shown in Table 3. The correlation

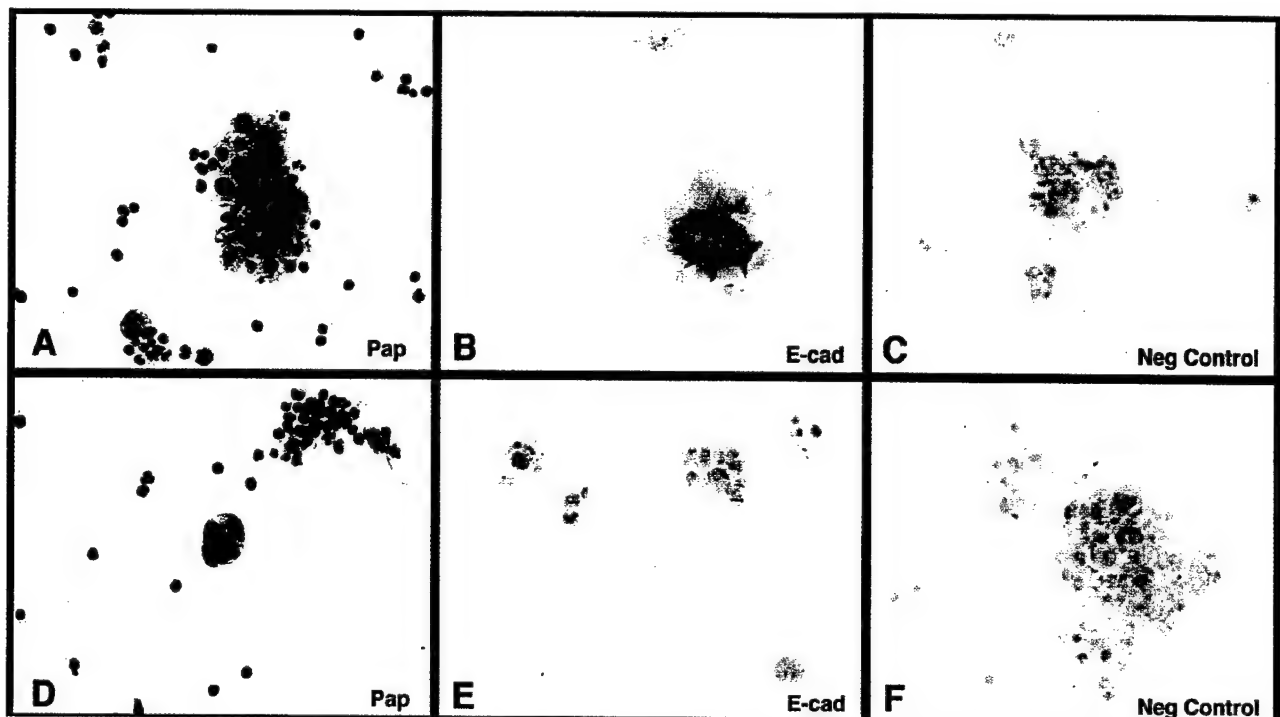


FIGURE 2. An example of a difficult case. A morphologic diagnosis of "atypical" was made on this case. A single cluster shows bright staining (B) and "atypical" morphology corresponding with a positive follow-up surgical specimen. Papanicolaou (Pap) stained examples (A and D), E-cadherin (E-cad) staining (B and E) and negative controls (neg control) (C and F) are as indicated. Note that some atypical-appearing mesothelial cells were negative for E-cadherin expression (E) suggesting they were reactive mesothelial cells (original magnification $\times 400$).

TABLE 2
Summary of E-Cadherin Scoring for All Cases

Cytologic diagnosis	E-cadherin positive	E-cadherin negative
Positive for malignancy	28	4
Negative for malignancy	3 ^a	7
Atypical/suspicious	2 ^b	1

^a Two of these three cases had borderline ovarian tumors after surgery.

^b Both of these cases had malignant tumors found during surgery.

of malignancy with staining for E-cadherin was tested using the chi-square method and had a value of 18.4, which suggests a highly significant association ($P < 0.0001$).

DISCUSSION

E-cadherin is highly specific for epithelial cells, but has been termed a tumor (or invasion) suppressor protein¹³ because it is lost in some malignancies.¹⁴ More than 50 articles have been published showing alterations in E-cadherin expression in many types of cancer, but true documented mutations are relatively less

TABLE 3
Final Tabulation of Staining, Based on Final Obtainable Diagnosis

Final diagnosis	E-cadherin positive	E-cadherin negative
Benign (8)	1	7
Malignant (37)	32	5

common. Specifically, lobular carcinoma of the breast^{11,15} and poorly differentiated gastric carcinomas¹² show mutations in the E-cadherin gene. The majority of other tumors show a reduction or alteration rather than a complete loss. The results of the current study confirm this expression pattern, in that most cases show retained E-cadherin expression, although its localization sometimes is altered. Other work in progress in the study laboratory also confirms this expression pattern. The authors found that only 1 of 81 ductal carcinomas of the breast truly lost expression, although $>80\%$ of the cases showed some alteration in staining (Dillon et al. unpublished data). In spite of many articles describing a reduction or alteration of expression, true loss of expression is rare.

Its expression, albeit altered or reduced, is useful for the diagnosis of carcinoma in fluids.

In this study there were four tumors that did not stain with the E-cadherin antibody. One of those was a melanoma, which was not expected to express E-cadherin. A second was a hepatocellular carcinoma. This may be due to true mutation because there is evidence in the literature of E-cadherin gene mutations in some cases of hepatocellular carcinoma.¹⁶ Finally, two other cases of metastatic adenocarcinoma failed to stain with E-cadherin antibody. These results most likely are simply examples of poorly differentiated tumors that have lost or have dramatically reduced expression of E-cadherin such that it is not detectable in the assay used by the authors.

Conversely, the authors were able to detect staining in four cases that were not clearly cytologically malignant. In each of these cases, the patients had ovarian tumors. This is consistent with the literature suggesting that serous ovarian tumors are among the most difficult to distinguish from reactive mesothelial cells. Notably, these are well differentiated tumors and E-cadherin loss is very rare in these tumors.¹⁷

Given these findings, it would be desirable to test the sensitivity and specificity of E-cadherin staining. In this primary study, the authors focused on the collection of a range of specimens to find cases in which E-cadherin did not stain the tumor cells. As a result, the cases chosen are not representative of a population of expected cases, and thus sensitivity and specificity statistics may not be valid. A new study is currently underway using sequential cases to assess sensitivity and specificity and compare this method with conventional cytomorphologic diagnoses.

Ultimately, other cadherins, especially N-cadherin may be useful in distinguishing mesothelial cells from tumors in effusions. N-cadherin has been shown to be useful in distinguishing adenocarcinomas from mesotheliomas because it is expressed in mesothelial cells, both benign and malignant, and not in epithelial cells.¹⁰

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Localization and Quantitation of Expression of the Cell Motility-Related Protein Thymosin β 15 in Human Breast Tissue

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Thymosin β 15 is a newly discovered 5300-Da protein that binds actin monomers and inhibits actin polymerization and might thus increase cellular motility. Thymosin β 15 is upregulated at both the mRNA and protein levels in prostate cell lines in a manner directly related to their capacity to metastasize. We hypothesize that because this protein is upregulated in cells with a propensity to metastasize, it might be a useful prognostic marker in breast cancer. Because this is a newly described protein, neither the subcellular localization of thymosin β 15 or its expression in breast cancer has been examined. We describe the use of an affinity-purified polyclonal antibody to show that within breast epithelium, thymosin β 15 is localized diffusely throughout the cytoplasm and that thymosin β 15 is upregulated in malignant (compared with benign) breast tissue. In contrast to the prostate model, thymosin β 15 is upregulated in nonmetastatic breast cancer and even ductal carcinoma *in situ* (compared with benign breast tissue), and, consequently, it might represent a potential early marker for breast malignancy. Additional studies are needed to evaluate the precise role and prognostic value of thymosin β 15 in breast cancer.

KEY WORDS: Actin, Carcinoma, Cytoskeletal, Immunoperoxidase, Mammary.

Mod Pathol 1997;10(11):1106-1112

β thymosins are a family of closely related, highly polar, 5-kDa polypeptides. All of the vertebrates studied and some invertebrates are known to contain one or often two β thymosins (1). Thymosin β 15 was recently uncovered in a search for proteins with increased expression in motile, as compared with poorly motile, Dunning rat prostatic carcinoma cell lines (2). The protein, which is 5300 Da, was designated "thymosin β 15" because of its approximately 60% homology with other members of the β thymosin family.

Thymosin β 4 is the most abundant β thymosin in most mammalian tissue and is the best studied member of this family. Current understanding is that thymosin β 4 sequesters a large pool of monomeric actin that is accessible to be released as needed for polymerization of actin filaments (3-5). Microinjection or overexpression of thymosin β 4 causes disassembly of actin stress fibers (6-8). Like thymosin β 4, thymosin β 15 binds monomeric actin and inhibits actin polymerization, abilities that confirm its place in the β thymosin family. Thymosin β 15 can also positively regulate cell motility, because transfection of antisense thymosin β 15 into motile rat prostatic carcinoma lines impairs cell motility in a Boyden chamber apparatus (2).

The putative role of β thymosins in modulation of the actin cytoskeleton through monomer sequestration suggests that they might be involved in cell differentiation, carcinogenesis, and metastasis. In some but not all cell lines, increased thymosin β 4 protein or mRNA correlated with differentiation (9). In human tumors, thymosin β 4 mRNA is increased in hairy cell leukemia and decreased in some lymphomas (10). Two of three metastatic colorectal carcinomas showed decreased thymosin β 4 mRNA,

0893-3952/97/010011-1106\$3.00/0 MODERN PATHOLOGY
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VOL. 10, NO. 11, P. 1106, 1997 Printed in the U.S.A.

Date of acceptance: August 4, 1997.

This work was supported, in part, by a grant from the Patrick and Catherine Weldon Donaghue Foundation for Medical Research (to DLR), by Grant DAMD17-94-J4366 from the United States Army Breast Cancer Research Program (to DLR), and Grant CA-37393 from the National Institutes of Health (to BRZ).

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compared with nonmetastatic tumors, with the third metastatic tumor showing little change (11). Thymosin β 10 mRNA levels are increased in renal cell carcinomas (12, 13), and thymosin β 10 upregulation correlated with the metastatic potential of melanomas (14). The expression of each thymosin β family member is independently regulated. Consequently, different family members might be independently increased or decreased in particular tumor types.

Because thymosin β 15 has only recently been described, it is less well characterized. It was shown to be upregulated in metastatic human prostate cancers at both the mRNA and protein level, when compared with less metastatic prostate cancers (2). Immunostaining of human prostate cancers revealed a general correlation between Gleason grade and thymosin β 15 expression, with high-grade tumors (Gleason Grade 8–10) showing more staining than did low-grade tumors (Gleason Grade 2–5).

The promising preliminary data from the prostate studies prompted us to undertake a study of thymosin β 15 expression in human breast tissue. Our aim was to assess the tissue and cellular localization of thymosin β 15 and to evaluate whether thymosin β 15 expression is associated with malignant changes of the breast epithelium. To do so, breast tissue samples representing a range of conditions from normal to neoplastic were examined using affinity-purified polyclonal anti-thymosin β 15 antibodies. We describe a significant association of high levels of expression of this protein with breast malignancy.

MATERIALS AND METHODS

Patients and Tumor Specimens

Forty-two formalin-fixed, paraffin-embedded tissue blocks were obtained from patients who underwent breast biopsies or breast resections between 1989 and 1996 at Yale–New Haven Hospital, New Haven, Connecticut. Twenty-one blocks of specimens from breast biopsies that yielded a benign primary diagnosis were selected, and an additional 21 blocks from resections or biopsies that yielded a malignant diagnosis of either ductal carcinoma *in situ* (DCIS), infiltrating ductal carcinoma, or infiltrating lobular carcinoma were selected. Clinical information corresponding to the 42 blocks was obtained from the Yale–New Haven Hospital pathology database.

Antibody

An affinity-purified polyclonal antibody raised against the 11C-terminal amino acids of thymosin

β 15 was used. The preparation and purification of this antibody was previously described (2).

Immunohistochemical Analysis

Standard histologic sections were cut from the paraffin blocks, baked overnight at 60° C, and deparaffinized. Slides were then soaked in 0.75% hydrogen peroxide in methanol to quench endogenous peroxidases. For antigen retrieval, each slide was immersed for 5 minutes in 6.5 mM sodium citrate, pH 6.0, in a heated conventional pressure cooker (15). After a 1-hour incubation in 0.3% bovine serum albumin diluted in Tris-buffered saline (TBS), pH 8.0 (Sigma, St. Louis, MO), for the slides stained by immunofluorescence or normal serum from the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) diluted in TBS, pH 8.0, to block nonspecific binding, the slides were incubated for 1 hour with the primary anti-thymosin β 15 antibody, diluted 1:100 in the solution used for blocking. The slides were then washed seven times. The slides were then incubated for 1 hour with secondary antibody. Cy3-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch Labs, West Grove, PA), diluted 1:500 in TBS, pH 8.0, was used as the secondary antibody for the immunofluorescent specimens, and biotinylated goat/anti-rabbit immunoglobulin G, diluted 1:200 in TBS, pH 8.0, was used for the peroxidase-stained specimens. The slides were then washed seven times. The peroxidase-stained slides were incubated 30 minutes in the Vectastain ABC reagent (Vector) prepared in TBS, pH 8.0, which is composed of avidin and biotinylated horseradish peroxidase, and then washed for 5 minutes in TBS, pH 8.0. All of the incubations were accomplished by coating the tissue with solution and storing the slides in a humidity chamber to prevent evaporation. The peroxidase-stained slides were then developed using the Vector 3,3'-diaminobenzidine kit (Vector) for 6 minutes, which uses diaminobenzidine and hydrogen peroxide. The peroxidase-stained slides were counterstained with hematoxylin. The immunofluorescent slides were coverslipped with *N*-propyl gallate and sealed with nail polish, and the peroxidase-stained slides were coverslipped with Immuno-mount (Shandon, Pittsburgh, PA). The immunofluorescent slides were stored at –20° C to preserve the signal, and the peroxidase-stained slides were stored at room temperature. Negative controls were prepared by substituting the primary antibody with blocking solution in each protocol.

Histologic Scoring and Analysis

The immunofluorescent slides were examined by two individuals (JSG, DLR) on an Olympus AX-70

(Melville, NY) epifluorescence photomicroscope, and representative photographs were obtained. For the peroxidase-stained specimens, each slide was examined by three observers (JSG, RADG, DLR), using a standard light microscope. For each slide, each observer assigned up to five scores representative of different pathologic elements contained on that slide. Each slide was scored for the staining of normal glands, hyperplastic glands, all benign cells, DCIS, and infiltrating cancer. The scores were based on the average staining of all the cells representing that diagnosis on the slide. Cells were scored as 0 if the staining was no greater than background, 1+ if staining was slightly greater than background, 2+ for an intermediate level of staining, and 3+ for very intense staining. If the observer deemed there to be insufficient or no tissue representing a particular diagnosis, a score was not assigned for that slide. On the basis of the scores from the three observers, a summary score was assigned for each element on each slide by totaling the scores if all three observers assigned scores. If only two observers assigned scores, the sum was multiplied by 3/2 to bring the summary score to the correct scale. A summary score was not given if one or no observers scored a particular diagnosis on a slide. Data analysis was accomplished using StatView 4.5 for Macintosh (Abacus Concepts, Berkeley, CA).

A total score (rather than a consensus score) was computed for each case to reflect the input of the three observers. The fact that the scores largely agreed lent validity to the study. In no case was there major discrepancy in scoring, *e.g.*, a score of 0 by a one reviewer and 3+ by another. Only two (3%) of the cases had two-point discrepancies (1+ *vs.* 3+). The remaining 97% of the cases either were scored identically by all of the three reviewers or had a one-point discrepancy, *e.g.*, two reviewers gave the case a 3+, whereas the third scored it as a 2+). Given this high degree of consensus, we were tempted simply to assign consensus scores, but we decided that preserving all of the scores gave a more accurate picture of the distribution of the staining pattern.

Optimally, we could score each case as positive or negative for expression, but because the level of expression is scored subjectively, we used a continuous scoring system. The rationale for using the cutoff of five to separate the positive from the negative cases is twofold. The first rationale for this division is theoretical. Because three-point discrepancies never occurred and two-point discrepancies were very rare, a case had to be scored as having at least intermediate staining by at least two reviewers to obtain a score of five. This seemed like a reasonable criterion to consider a case as positive. The second rationale for this division is that the score of five would be extremely sensitive for a diagnosis of malignancy, with 94% of the malignant cases scoring five or higher and only one case scoring below

five. Also, the peak of the distribution for the benign cases lies below five, with 65% of the cases scoring less than five. Ultimately, we found that using five as a cutoff does indeed provide the best separation between the benign and malignant cases.

RESULTS

Localization

Paraffin-embedded tissue from both benign and malignant breast lesions was stained by the immunofluorescence method to evaluate the subcellular localization of thymosin β 15 and the relative specificity of the affinity-purified anti-thymosin β 15 antibody. In all of the breast epithelial cells, staining for thymosin β 15 was located diffusely throughout the cytoplasm and was excluded from the nucleus (Fig. 1). The intensity of the staining of breast epithelial cells varied from slide to slide and even within the same slide. Although the intensity of the staining was not formally scored in the slides stained by the immunofluorescence method, it was clear that there was at least one example for both benign and malignant glands where there was intense staining and at least one example for each of staining that was not significantly greater than background. The anti-thymosin β 15 antibody stained occasional endothelial cells in addition to the breast epithelial cells. Also, in some slides, it seemed to stain more intensely in myoepithelial cells (Fig. 1), but this finding was not represented in all of the specimens.

Expression

For ease of interpretation, the remainder of study was done using conventional peroxidase-based staining and a standard light microscope. Forty-three slides of surgical specimens representing a spectrum of breast lesions were stained using the anti-thymosin β 15 antibody and visualized with a biotinylated secondary antibody, peroxidase-linked avidin, and diaminobenzidine substrate. Twenty-one slides had primary diagnoses that represent benign changes, and 22 slides had a primary diagnosis of malignancy (6 DCIS, 16 infiltrating cancer). Many slides contained tissue representing more than one diagnosis, but there were no malignant changes on any of the slides for which the final diagnosis was benign and no infiltrating cancer on slides from cases with the final diagnosis of DCIS. All of the slides were from female patients, and the average age of the patients was 43 years for the benign slides and 59 years for the malignant slides.

As with the immunofluorescent slides, there was some minimal background staining of the stroma. There were variable levels of thymosin β 15 expression between the slides, such that the level of stain-

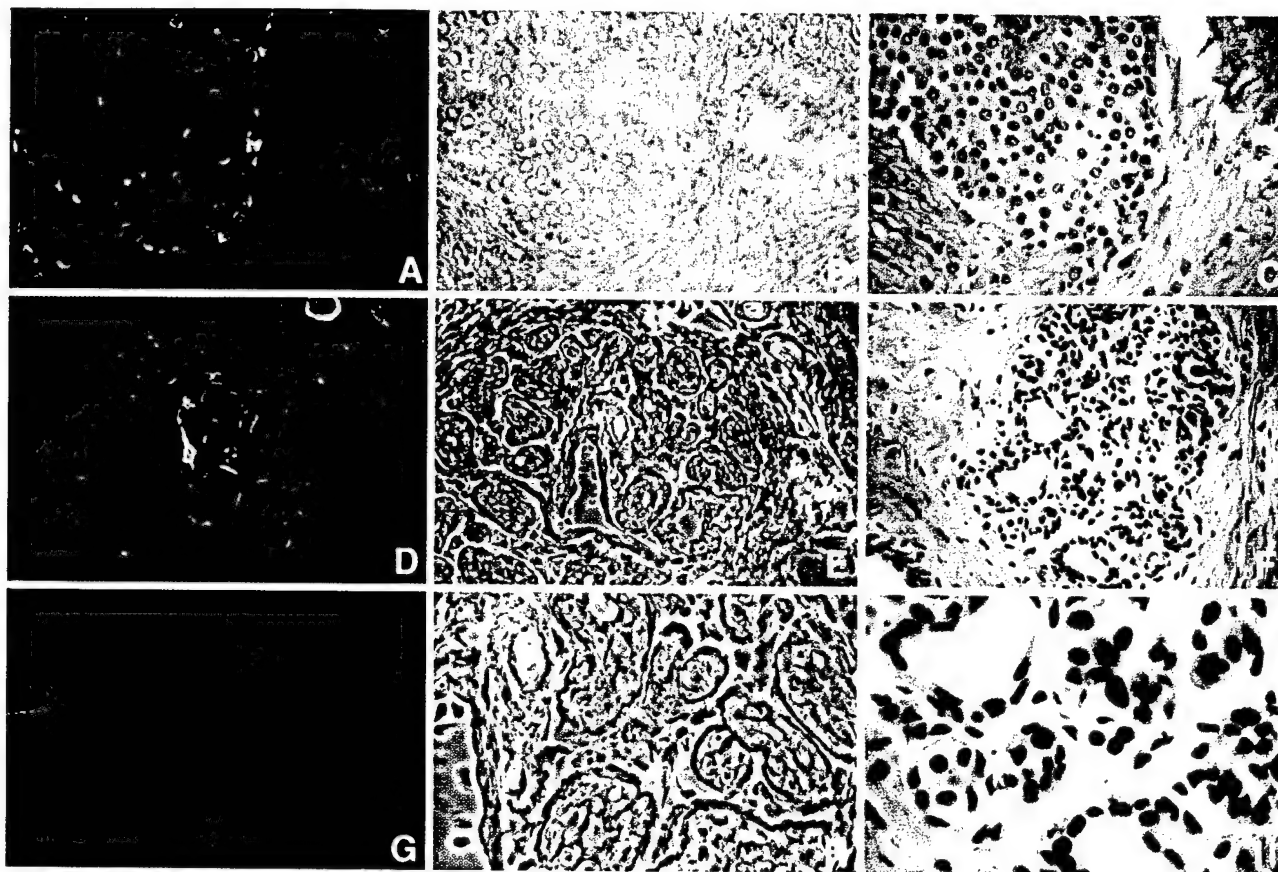


FIGURE 1. Expression of thymosin $\beta 15$ in breast tissue. **A**, the expression pattern of thymosin $\beta 15$ in infiltrating ductal carcinoma is visualized by staining with an affinity-purified polyclonal antibody directed against thymosin $\beta 15$ followed by a Cy3 conjugated goat anti-rabbit secondary antibody. Thymosin $\beta 15$ is localized diffusely throughout the cytoplasm in the tumor cells but is excluded from the nucleus. **B**, the corresponding phase image of the same field. **C**, the same structure in a serial section stained with hematoxylin and eosin. **D**, an immunofluorescent image of the myoepithelial cell staining pattern for thymosin $\beta 15$. **E**, corresponding phase. **F**, hematoxylin and eosin stain. Original magnification, 200 \times for A-F, 400 \times for G-I.

ing could be scored with relation to background. Each of the slides was examined and scored by three people. Examples of the different scores are shown in Figure 2. The three individual scores were compiled to give combined scores on a scale from 0 to 9. Table 1 shows a summary of the benign cases, including the diagnosis and overall score. Table 2 contains similar data for the malignant cases, but, in addition to the overall score of the benign elements, it also includes scores for the DCIS and invasive cancer regions on each slide. For the slides with infiltrating cancer, the status of the axillary lymph nodes with respect to metastases, the size of the resected tumor, the estrogen and progesterone receptor status, and the ploidy of the tumor are shown.

A summary of all of the data separated by diagnosis is provided in Table 3. The average score shown represents the score for the combined elements for those slides with a benign primary diagnosis, for the DCIS on the DCIS slides, and for the infiltrating cancer on the slides with infiltrating cancer. The score for each slide was converted to a binary value of either positive (scores ≥ 5) or negative (scores < 5). The percentage of slides in each

diagnosis scored as positive is also shown in Table 3. The distribution of the scores by tissue type is shown in Figure 3, illustrating the preponderance of stronger staining in malignant epithelium than in benign epithelium and justifying the division point for the binary scoring.

To evaluate these data for significance, a contingency table was constructed (Table 4). Calculation of the χ^2 statistic shows a statistically significant increase in the number of malignant slides scored positive, compared with the benign slides ($P = .0002$). There were also more DCIS slides scored as positive ($P = .0149$) and infiltrating cancer slides scored as positive ($P = .0014$) when these were compared individually to the benign slides. The slides of tumors with and without nodal metastasis were both more positive than the benign slides when compared individually ($P = .0329$ and $P = .0311$, respectively). Among the selected slides, there were nine cases that allowed the comparison between malignant cells and benign cells from the same patient (on the same slide). In this group, the malignant cells received more positive scores than the adjacent benign cells ($P = .0034$). No correlation was found in staining pattern with age, node

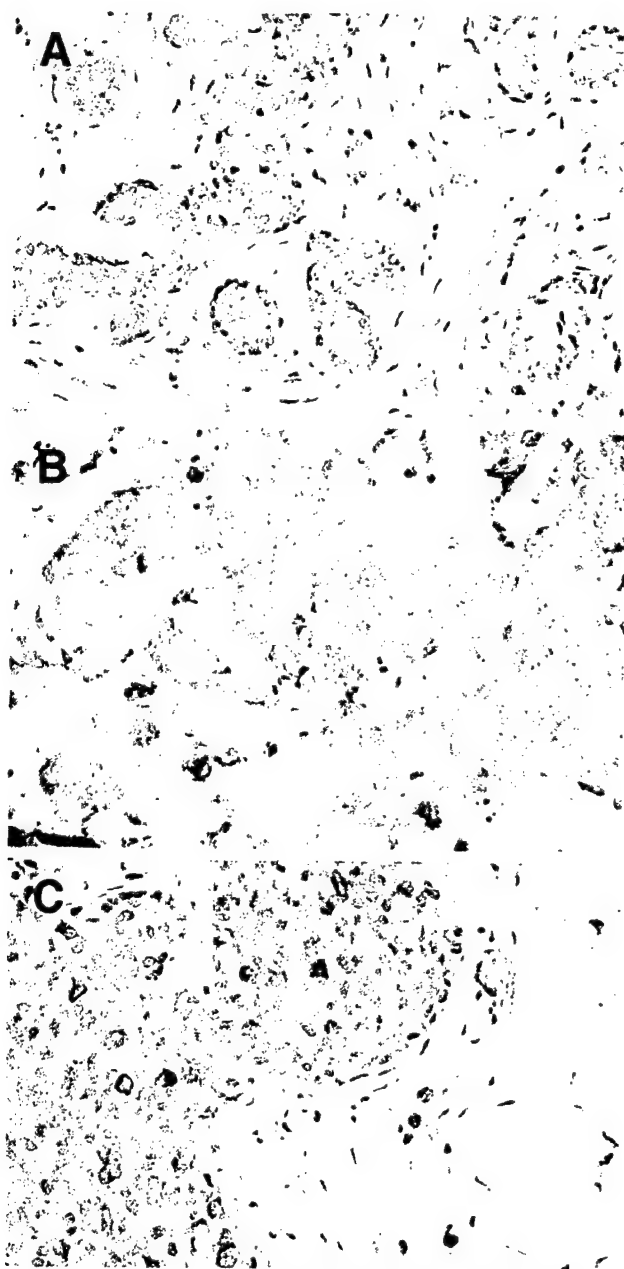


FIGURE 2. Variation in the intensity of staining of thymosin $\beta 15$ in breast epithelial cells. Histologic sections were stained with an affinity-purified polyclonal antibody directed against thymosin $\beta 15$ and visualized with a peroxidase/3,3'-diaminobenzidine method. **A**, expression of thymosin $\beta 15$ in benign breast ducts that is slightly greater than background. **B**, an intermediate expression level as seen in an infiltrating ductal carcinoma. **C**, a very intense staining pattern is seen in another case of infiltrating ductal carcinoma. These figures illustrate the scoring system defined for thymosin $\beta 15$ expression in breast epithelial cells. The level of thymosin $\beta 15$ expression was scored as either 1+ (**A**), 2+ (**B**), or 3+ (**C**). Negative controls with goat serum only were used as a baseline reference for each case.

status, size, estrogen receptor status, progesterone receptor status, or ploidy.

DISCUSSION

Thymosin $\beta 15$, like other members of the β thymosin family, is known to bind actin monomers

TABLE 1. Summary of Clinical Data and Thymosin $\beta 15$ Staining of Benign Cases

Primary diagnosis	Age (yr)	Score
Fibroadenoma	36	6.0
	34	4.0
	24	4.0
	58	3.0
	18	7.0
	25	4.0
	37	2.0
	27	8.0
	60	3.0
Fibrocystic disease	40	3.0
Sclerosing adenosis	53	3.0
	56	6.0
	42	5.0
	43	3.0
	60	4.0
Atypical hyperplasia	54	7.0
	44	5.0
	29	—
	46	3.0
	57	4.0
	63	3.0

and is thought to be associated with cell motility. Evidence that it is upregulated in human prostate cancers and that upregulation seems to correlate with disease severity suggests that it might mark metastatic potential (2). This study suggests that breast tissue might show similar properties, *i.e.*, upregulation is associated with malignant changes of the ductal epithelium. Although the definition of progressive stages of severity in breast cancer is less well quantitated than it is in prostatic cancer, both DCISs and infiltrating ductal carcinomas contain more thymosin $\beta 15$ than does benign breast tissue. One measure of severity or progression in breast cancer is metastasis. In this study, seven of eight tumors with nodal metastases at presentation showed positive staining, consistent with upregulation of this protein in association with motility. Ours is a pilot study, which has too few samples for a quantitative assessment of this trend.

Although less well defined than progression in colorectal carcinoma, it is generally presumed that there is a progression from DCIS to nonmetastatic infiltrating ductal carcinoma to metastatic breast cancer. Both DCIS and node-negative breast cancer have increased thymosin $\beta 15$ expression in this study. Thus, it seems that upregulation of thymosin $\beta 15$ could occur early in the oncogenic pathway.

In this study, some malignant lesions stained weakly or moderately, whereas some of the benign tissue seemed to have a strong staining pattern. We are unsure of the significance of this observation. It is conceivable that there is a wide variation in thymosin $\beta 15$ expression within a given lesion. Although there are examples of benign tissue with increased expression and malignant tissue with

TABLE 2. Summary of Clinical Data and Thymosin β 15 Staining of Malignant Cases

Primary diagnosis	Age (yr)	Node status	Tumor size	ER status	PR status	Ploidy	Benign score	DCIS score	Invasive cancer score
DCIS	53	N/A	N/A	N/A	N/A	N/A	4.0	8.0	N/A
	25	N/A	N/A	N/A	N/A	N/A	3.0	5.0	N/A
	46	N/A	N/A	N/A	N/A	N/A	5.0	—	N/A
	83	N/A	N/A	N/A	N/A	N/A	4.5	6.0	N/A
	41	N/A	N/A	N/A	N/A	N/A	3.0	5.0	N/A
Ductal carcinoma	65	N/A	N/A	N/A	N/A	N/A	4.0	7.0	N/A
	69 ^a	Positive	3.0	Negative	Negative	Diploid	—	—	9.0
	69 ^a	Positive	3.0	Negative	Negative	Diploid	3.0	—	—
	45	Positive	10.0	Negative	Negative	Unknown	3.0	7.0	7.0
	44	Positive	2.5	Unknown	Unknown	Unknown	—	7.0	6.5
	38	Positive	Unknown	Positive	Negative	Aneuploid	3.0	—	3.0
	64	Positive	4.5	Negative	Negative	Unknown	—	6.0	7.5
	74	Negative	5.5	Positive	Positive	Unknown	—	—	5.0
	74	Negative	1.0	Unknown	Unknown	Unknown	—	9.0	8.0
	45	Positive	2.0	Negative	Negative	Diploid	9.0	9.0	9.0
	84	Negative	2.0	Unknown	Unknown	Unknown	—	9.0	9.0
	74	Positive	3.0	Unknown	Unknown	Aneuploid	4.5	6.0	5.0
	67	Positive	3.0	Negative	Negative	Aneuploid	—	6.0	6.0
	43	Negative	1.5	Negative	Negative	Aneuploid	—	—	9.0
	78	Negative	1.0	Unknown	Unknown	Unknown	3.0	—	—
Lobular carcinoma	71	Unknown	3.0	Negative	Positive	Unknown	—	9.0	9.0
	40	Negative	4.0	Negative	Positive	Diploid	5.0	—	—

DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; PR, progesterone receptor; N/A, not applicable.

^a These two slides represent tissue from the same tumor. On one slide, the benign glands were poorly preserved and not scored, whereas on the other slide, the invasive cancer was poorly preserved and not scored.

TABLE 3. Staining of Slides for Thymosin β 15 Grouped by Diagnosis

Primary diagnosis	Number of slides	Number not scored	Average score	Percentage positive
Fibroadenoma	9	0	4.6	33
Fibrocystic disease	1	0	3.0	0
Sclerosing adenosis	5	0	4.2	40
Atypical hyperplasia	6	1	4.4	40
All benign	21	1	4.3	35
DCIS	6	1	6.2	100
Infiltrating ductal carcinoma	15	2	7.1	92
Infiltrating lobular carcinoma	1	1	—	—
All infiltrating cancer	16	3	7.1	92
All malignant	22	4	6.9	94
Total	43	5	5.5	70

DCIS, ductal carcinoma *in situ*.

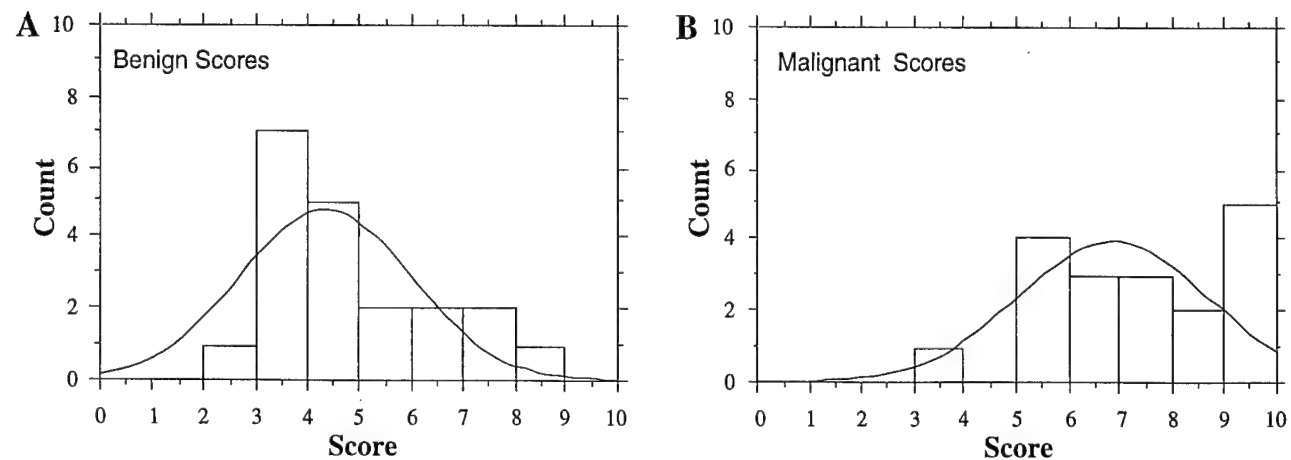


FIGURE 3. Distribution of cases by score of thymosin β 15 staining in benign (A) and malignant (B) breast tissue. The slides were scored by three observers for intensity of staining of the breast epithelium, and the combined scores are shown grouped by benign ($n = 20$, histogram A) or malignant ($n = 18$, histogram B) primary diagnoses. Each bar on the histograms represents the number of cases with a score that is greater than or equal to the number on the axis at its left margin and less than the number on the axis at its right margin. Each curve represents a normal distribution with the same mean and standard deviation as the cases on the histogram. The histograms show that the score of 5 seems to separate the benign from the malignant cases, with the majority of benign cases scoring less than 5 and all but one of the malignant cases scoring 5 or greater.

TABLE 4. Contingency Table of Benign or Malignant Slides versus "Positive" or "Negative" Staining for Thymosin β 15

	Negative	Positive	Totals
Benign	13	7	20
Malignant	1	17	18
Totals	14	24	38

$\chi^2 = 14.387$; Fisher's exact P value = .0002.

baseline expression, the overall trend of malignancy was toward increased thymosin β 15 expression. It is also likely that increased levels are associated with physiologic epithelial remodeling. Because the breast is an organ that undergoes continual remodeling (associated with the menstrual cycle), it is not surprising to see this that motility-related protein is expressed in some benign tissues. Expression in that context could obscure the changes associated with malignancy.

The diagnostic utility of the increased thymosin β 15 expression seen in malignancy remains to be assessed. This work, though provocative, has insufficient numbers and follow-up to evaluate the independent prognostic value of this marker. Work is underway to produce a monoclonal antibody that will be used in future studies. In summary, this study shows that within breast epithelium, thymosin β 15 is localized to the cytoplasmic compartment, as is the case with thymosin β 4 and thymosin β 10 (8), and that thymosin β 15 is upregulated in malignant (compared with benign) breast tissue. Future studies are planned to evaluate the precise role and prognostic value of thymosin β 15 in breast disease.

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A Mutation In α -Catenin Disrupts Adhesion In Clone A Cells Without Perturbing Its Actin And β -Catenin Binding Activity

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(Received 16 July 1997; In final form 7 August 1997)

Cadherin mediated cell-cell adhesion requires cytoplasmic connections to the cytoskeleton mediated by α -catenin. Original descriptions of the catenins, as well as our own in vitro studies, have suggested that this connection was mediated by the interaction of α -catenin to actin. Loss of adhesion in the human colon carcinoma cell line "Clone A" is the result of an internal deletion mutation of 158 residues near the N-terminus of the protein resulting in an 80 kD mutated protein. Transfection of these cells with the full length protein restores the normal adhesive phenotype. We have characterized this mutant protein in efforts to understand the normal function of α -catenin and, in particular, the region deleted in the Clone A mutant. Co-precipitation experiments using whole cell lysates indicate that the mutant form of α -catenin binds β -catenin and plakoglobin, and can form a structural complex with E-cadherin via these interactions. Actin co-sedimentation assays show that the recombinant mutant binds and bundles F-actin and binds both actin and β -catenin simultaneously, as seen with wild type α -catenin. These results suggest that the stabilization of the E-cadherin-catenin complex may be mediated by factors beyond its direct interaction with actin. We conclude that a region near the N-terminus of α -catenin mediates additional interactions between the adhesive complex and the cytoskeleton that are critical for functional adhesion.

Keywords: E-cadherin, cytoskeleton, plakoglobin, adherens junction, carcinoma

INTRODUCTION

Loss of calcium dependent E-cadherin mediated cell-cell adhesion is associated with metastasis and tumor invasion in epithelial cells (Behrens, et al., 1989; Birchmeier, et al., 1993; Frixen, et al., 1991; Vleminckx, et al., 1991). Multiple mechanisms for loss of

adhesion have been described, including inadequate E-cadherin expression (Bringuier, et al., 1993; Doki, et al., 1993; Dorudi, et al., 1993; Hashimoto, et al., 1989; Mayer, et al., 1993; Oka, et al., 1993; Shimoyama and Hirohashi, 1991), mutations in the E-cadherin gene (Becker, et al., 1994; Berx, et al., 1995; Kanai, et al., 1994) and the inability of the catenins to

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anchor E-cadherin to the cytoskeleton (Kadowaki, *et al.*, 1994; Oda, *et al.*, 1993; Sommers, *et al.*, 1994). The connection of E-cadherin to the cytoskeleton, via the catenins, is also a potential target for mutations that result in loss of adhesion and this has been described for both α -catenin (Morton, *et al.*, 1993; Shimoyama, *et al.*, 1992) and β -catenin (Kawanishi, *et al.*, 1995; Oyama, *et al.*, 1994).

A model has evolved to describe the interactions between cadherins and catenins that establish a functional adhesion complex. The binding order of these protein-protein interactions have been biochemically defined (Aberle, *et al.*, 1994; Ozawa and Kemler, 1992) and it is thought that E-cadherin binds to β -catenin which in turn binds to α -catenin (Jou, *et al.*, 1995). It is known that α -catenin is associated only indirectly with E-cadherin via its interaction with either β -catenin or plakoglobin (Aberle, *et al.*, 1996; Sacco, *et al.*, 1995), but not both simultaneously (Butz and Kemler, 1994). The fourth catenin, p120CAS (Reynolds, *et al.*, 1994), also appears to bind to E-cadherin but does not appear to compete for the same site on the cadherin molecule that binds to β -catenin and plakoglobin. Also, p120CAS does not bind to α -catenin (Daniel and Reynolds, 1995). Alpha-catenin can bind directly to F-actin (Rimm, *et al.*, 1995) thereby completing one possible linkage of E-cadherin to the cortical cytoskeleton, as required for functional adhesion. Recently α -catenin has also been shown to interact with other proteins. The interaction with α -actinin (Knudsen, *et al.*, 1995) has been published and preliminary data on interactions with vinculin and ZO-1 (Nagafuchi, *et al.*, 1996), spectrin (Lombardo, *et al.*, 1994) and itself (as a dimer) (Koslov, *et al.*, 1996) have been presented in abstract form. The role of all of these interactions in adhesion remains to be determined.

Even though a simple model has been proposed where the cadherin-catenin complex is linked to the cytoskeleton through the interaction of α -catenin with actin (Rimm *et al.*, 1995), newly identified interactions suggest a broader role for α -catenin. Recent works have described participation of α -catenin in at least two adhesive states. Construction of E-cadherin- α -catenin chimeras showed triton insolubility for both

N- and C-terminal catenin chimeras, even though only the C-terminal chimera produced strong adhesion (Nagafuchi, *et al.*, 1994). The same system was used to suggest that two adhesive states exist (Takeda, *et al.*, 1995). This concept was confirmed and extended by recent studies with C-cadherin which quantitated strong and weak adhesive states (Briher, *et al.*, 1996). These findings all suggest there may be other critical connections to the cytoskeleton, beyond those in the simple model.

One mechanism to address the range of functional connections between α -catenin, the cytoskeleton, and the adhesion complex is examination of *in vivo* mutations that result in loss of adhesive function. We hypothesize that if the connections of α -catenin with actin and β -catenin are critical for productive adhesion, then mutations that cause loss of adhesion must be localized to, or otherwise affect, these sites of interaction. This work describes a mutation in the α -catenin gene that abrogates calcium dependent cell-cell adhesion in the human colon carcinoma cell line "Clone A". We have localized this mutation and examined its structural role in the adhesion complex in efforts to further understand the normal function of α -catenin and, in particular, the significance of the region deleted in the Clone A mutant. Surprisingly, we find that α -catenin's interactions with both actin and β -catenin are intact in this cell line, suggesting a broader model for the functional connection of α -catenin and the adhesive complex to the cytoskeleton or other integral membrane proteins.

METHODS

Cell Lines and Antibodies

The human colon carcinoma cell line "Clone A" (Breen, *et al.*, 1993) was provided by Dr. Arthur Mercurio, Deaconess Hospital, Harvard Medical School, Boston, MA. Clone A cells were maintained in RPMI-H 1640 supplemented with 10% fetal calf serum (FCS) and 0.3% L-Glutamine. The human colon carcinoma cell line HT-29 was provided by Dr. Xin-Yuan Fu, in our department. HT-29 cells were maintained in McCoy's modified medium with 10%

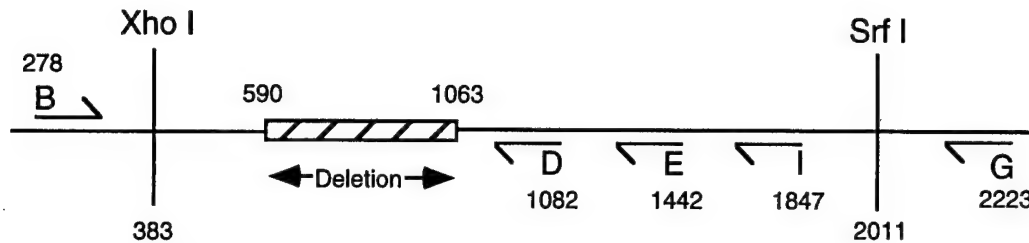


FIGURE 1 Construction of mutant α -catenin cDNA. A schematic showing the location of the restriction sites used for cloning and the primers used for RT-PCR and sequencing of the mutated form of α -catenin found in Clone A cells. Primers are named with letters and the number of the first nucleotide shows their position with respect to the α -catenin cDNA sequence (Rimm, et al., 1994).

FCS and 0.3% L-Glutamine. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Mouse monoclonal antibodies against human E-cadherin (TLE1) and mouse α -catenin (TL α 1), β -catenin (TL β 1) and Plakoglobin (TLP1) were purchased from Transduction Laboratories, Lexington, KY. Other mouse monoclonal antibodies to α -catenin (α 3H4, α 6A9, α 3C1, α 5B11, α 7A11, α 10E1) were produced in collaboration with Dr. Linda Bullions and Dr. Arnold Levine, Princeton University, NJ as described (Bullions, et al., 1997). The YR6 antibody is a polyclonal anti- β -catenin antibody which was produced as described (Koslov et al., 1997).

Plasmids

All recombinants and clones referred to hereafter in this paper as α -catenin are derived from the α_1 (E)-catenin isoform cDNA (GenBank accession #L23805, (Rimm, et al., 1994). Both the pGEX/ α -catenin and pGEX/ β -catenin plasmids were constructed as described (Rimm, et al., 1995). The pcDNA3/ α -catenin plasmid was constructed by cloning full length α -catenin cDNA (Rimm, et al., 1994) into the Eco RI site of the mammalian expression vector pcDNA3 (Invitrogen) containing the cytomegalovirus promoter and the neomycin-resistance gene. The recombinant Clone A α -catenin protein was prepared using the prokaryotic expression vector pGEX (Pharmacia). The pGEX/Clone A α -catenin was constructed as follows: Clone A α -catenin cDNA was synthesized from mRNA using reverse transcriptase

as described below. A PCR product containing the deleted region of the α -catenin gene was synthesized from Clone A cDNA using the primers B and G (Figure 1). The 1505 bp PCR product was purified from a 1% agarose gel using the GeneClean II kit (BIO 101 Inc.) and subjected to a double digest with restriction enzymes Xho I and Srf I which flank the deleted region. After digestion, the 1153 bp Xho I/Srf I fragment was gel purified and ligated into the full length α -catenin cDNA in the pGEX vector (pGEX/ α -catenin) (Rimm, et al., 1994) from which the wild type Xho I/Srf I fragment had been liberated. This created the plasmid pGEX-Clone A/ α -catenin which was screened for orientation and the region of the α -catenin cDNA containing the ligated fragment was sequenced using primers B, D, E, I, and G (Figure 1) to identify any errors introduced by PCR or the cloning procedure.

Transfection Procedure

5×10^5 cells were plated in 60 mm petri dishes and incubated overnight. The following day, cells were transfected with 3-6 μ g of the mammalian expression vector pcDNA3 (Invitrogen), harboring the full length α -catenin cDNA (pcDNA3/ α -catenin) using the lipofectamine reagent (Gibco/BRL), according to the manufacturer's protocol. Neomycin resistant clones were isolated by selective growth in medium containing 0.6 mg/ml of G418 (Gibco/BRL). Subclones were primarily identified on the basis of their phenotype change expected to be associated with expression of the full length α -catenin protein. Clones were isolated

using cloning rings and expression of full length α -catenin was verified by immunofluorescence.

Immunofluorescence

Cells were plated onto glass chamber slides and grown to subconfluence. Cells were washed 5 times in Tris buffered saline (TBS) pH 8.0 (50 mM Tris, 150 mM NaCl) and fixed in methanol for 20 minutes at 4°C. After fixation, cells were washed again and blocked with 0.3% BSA/TBS for 1 hour at room temperature. After blocking, cells were washed and incubated with various primary antibodies (diluted in 0.3% BSA/TBS) for 1 hour at room temperature. Antibodies were used at the following dilutions: monoclonal E-cadherin (TLE1) 1:50, polyclonal β -catenin (YR6) 1:250, monoclonal α -catenin (α 3H4) and (α 7A11) were used as undiluted culture supernatants. After incubation with the primary antibodies cells were washed and incubated with CY3 conjugated secondary antibodies (Amersham) diluted 1:500 in 0.3% BSA/TBS for 1 hour at room temperature. Cells were finally washed, mounted and viewed using an Olympus AX-70 fluorescent microscope.

Western Blot Analysis

Whole cell extracts were obtained from confluent cells by solubilizing directly with Laemmli sample buffer. Extracts were subjected to SDS-PAGE under denaturing conditions (Towbin, et al., 1979) followed by Western blot analysis using ECL detection reagents (Amersham) according to the manufacturer's protocol. Primary antibodies were used at the following dilutions: α -catenin (TL α 1) 1:1000, β -catenin (TL β 1) 1:1000.

Detergent Extraction of Cells

Cells were grown to confluence and extracted in situ with Triton X-100 using a shortened version of the protocol previously described (Devarajan, et al., 1994). Confluent cells were washed with ice cold PBS (pH 7.5) and extracted with buffer 1 (10 mM Pipes pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 0.5% Triton X-100, 1.2 mM PMSF, 0.5 mM Pefa-

blocSC (Centrachem) for 10 minutes on ice, yielding the detergent soluble or "cytoplasmic" fraction. Cells were then extracted with buffer 2 (identical to buffer 1 except that 250 mM Ammonium Sulphate was substituted for 100 mM NaCl) for 10 minutes on ice yielding the detergent insoluble or "cytoskeletal" fraction. Both fractions were diluted in Laemmli sample buffer and boiled for 5 minutes prior to SDS-PAGE and Western blot analysis.

Immunoprecipitation

One million cells were washed three times in ice cold PBS and lysed by adding 1 ml of lysis buffer (1% NP40, 1% Triton X-100, 1 mM CaCl₂, 2mM PMSF, 2mM Leupeptin, 2mM Aprotinin) in TBS (pH 7.6) and placing at 4°C for 1 hour. The cell lysates were centrifuged at 14,000 rpm for 15 minutes at 4°C and the supernatant was incubated with 50 μ l of Omnisorb reagent (Calbiochem) for 1 hour at 4°C. The cell lysate plus Omnisorb was centrifuged at 10,000 rpm for 40 seconds to pellet the Omnisorb reagent and the supernatant was incubated with 3 μ g of the immunoprecipitating antibody for 1 hour at 4°C. After an hour, 50 μ l of the Omnisorb reagent was added to the cell lysate/antibody mix and incubated for an additional hour at 4°C. The Omnisorb was pelleted by centrifuging at 10,000 rpm for 40 seconds and washed twice with 1 ml of wash buffer A (TBS pH 7.6, 0.1% Triton X-100, 0.15 M NaCl, 2 mM PMSF, 2 mM Leupeptin, 2 mM Aprotinin), twice with 1 ml of wash buffer B (TBS pH 7.6, 0.1% Triton X-100, 0.5 M NaCl, 2 mM PMSF, 2 mM Leupeptin, 2 mM Aprotinin) and once with 1 ml of wash buffer C (Tris pH 6.8, 2 mM PMSF, 2 mM Leupeptin, 2 mM Aprotinin). After washing, 20 μ l of Laemmli sample buffer was added to the Omnisorb pellet, mixed well and boiled for 5 minutes. The Omnisorb was pelleted by centrifuging at 10,000 rpm for 40 seconds and the supernatant subjected to SDS-PAGE and Western blot analysis.

RT-PCR Amplification

Total RNA was isolated from cell lines using the TRIzol LS reagent (Gibco/BRL). Subsequently,

cDNA was synthesized from 3 μ g of RNA using 2 μ M oligo-dT primer pd(T)₁₂₋₁₈ (Pharmacia) and 200U of Superscript II Reverse Transcriptase (Life Technologies) in reverse transcriptase (RT) buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂) containing 0.01 M dithiothreitol and 10 mM dNTP's. The RT reaction was incubated at 42°C for 1 hour. Alpha-catenin cDNA was amplified using the various primers as shown below.

Primers

Primers used for PCR and sequencing are shown below:

Cloning and Sequencing of PCR Products

The PCR product resulting from amplification of Clone A α -catenin cDNA using the A and D primers (Figure 4) was ligated into the PCR II vector of the TA cloning kit (Invitrogen). This PCR fragment contained the deleted region and was sequenced by the Keck sequencing facility at Yale using primers A, B, and D (Figure 4) with Taq FS DNA polymerase and fluorescently-labeled dideoxynucleotides in a thermal cycling protocol.

Actin Binding Assay

Rabbit skeletal muscle actin was polymerized by diluting in 20 mM Tris pH 7.2, 100 mM NaCl, 2 mM MgCl₂, 2.5 mM DTT and incubating for 30 minutes at room temperature. Polymerized actin (final concentration 6.5 μ M) was added to approximately 7-8 μ M of

a single recombinant GST-fusion protein in 20 mM Tris pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 2.5 mM DTT and allowed to interact for 30 minutes - 1 hour at room temperature. When two recombinant GST-fusion proteins were added to actin at the same time, 14-16 μ M (total protein) was used at approximately a 1:1 molar ratio and the proteins were allowed to interact with each other for one hour at room temperature before adding to the polymerized actin. The GST-fusion protein/actin mixtures were centrifuged at 10,000 xg for 30 minutes at room temperature and comparable amounts of supernatant and pellet fractions were subjected to SDS-PAGE and stained with Coomassie blue.

Synthesis of GST-Fusion Proteins

The GST- β -catenin, GST- α -catenin and GST-Clone A α -catenin fusion proteins were expressed from the pGEX/ β -catenin, pGEX/ α -catenin and pGEX/Clone A α -catenin plasmids in *E. coli*. Bacterial cultures were grown for 3 hours and then induced for 3 hours with isopropyl B-D-thiogalactopyranoside before harvesting. Lysis was achieved by four repetitions of a 30 second sonication on ice in TBSE (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA) with 1 mM DTT and 1 mM of each of the protease inhibitors PefablocSC, Chymostatin, Leupeptin, Antipain and Pepstatin (Sigma Chemical Co.). The lysate was centrifuged at 15,000 xg and the supernatant was affinity purified on glutathione-agarose (Sigma Chemical Co.) at 4°C and washed extensively with TBSE containing 0.1 mM DTT. Peptides were cleaved by the addition of thrombin (Sigma) in 20

name	direction	nucleotide position	sequence
A	forward	7-24	5'-GCTGTCCATGCAGGCAAC-3'
B	forward	261-278	5'-GGAGGAGCTTGTGGTTGC-3'
C	forward	590-607	5'-AATTGAAAGATGTTGGGC-3'
D	reverse	1100-1082	5'-GCAGAATTGAGTGCATCAC-3'
E	reverse	1459-1442	5'-GATCCATGTTCTCTGGG-3'
F	forward	2286-2305	5'-GACCATTCGAGACCATTGCC-3'
G	reverse	2241-2223	5'-GGCAGCACTGATGACATCC-3'
H	reverse	3346-3328	5'-GCATCGAGACACTGTAGCC-3'
I	reverse	1871-1847	5'-CGGATGCCATCAAATACCAGGCGGG-3'

mM Tris, 120 mM NaCl, 2.5 mM CaCl₂, 2.5 mM KCl at 20°C for 10 minutes. After digestion, thrombin was inactivated with 0.3 mM PMSF and the cleaved peptide was eluted in TBSE with 20 mM DTT added to the eluates. Eluted recombinant proteins were then dialyzed into Tris buffered binding buffer (20 mM Tris pH 7.5, 100 mM NaCl, 0.1 mM DTT) prior to use.

RESULTS

Identification and mapping of a mutant form of α -catenin in Clone A cells.

As Clone A cells had previously been reported to lack the α -catenin protein (Breen, *et al.*, 1993) an initial Western blot was conducted for confirmation. Using the anti- α -catenin antibody TL α 1, a predominant 80 kD band as well as the normal 102 kD band was seen in Clone A cell lysates (Figure 2). Since the TL α 1 antibody recognizes the 80 kD mutant form of α -catenin and this antibody maps to the carboxy-terminus of the protein (aa 729-755), we concluded

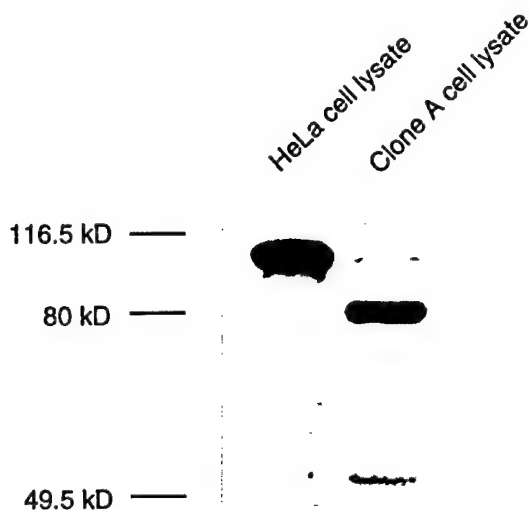


FIGURE 2 Western blot using a monoclonal antibody to α -catenin (TL α 1) shows the normal size (102 kD) protein on the left (HeLa cell lysate) and the mutated form (80kD) from Clone A cells on the right. Small amounts of wild type protein are also reproducibly visible in the Clone A lane.

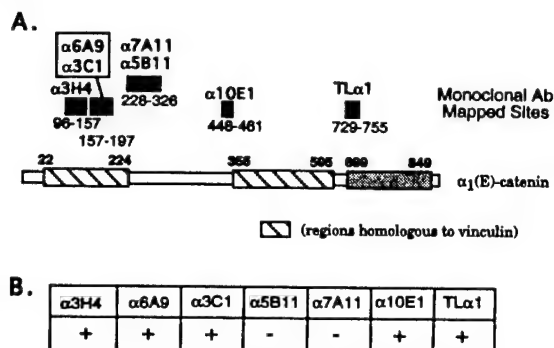


FIGURE 3 A schematic showing the mapped sites of various monoclonal antibodies to α -catenin relative to the regions of vinculin homology (A). A series of recombinant GST-fusion proteins were constructed and tested for antibody binding on Western blots to determine the location of monoclonal antibody binding (not shown). The antibodies are named above the filled boxes and the numbers shown beneath the boxes are the smallest region tested that binds antibody. The lower table (B) shows the reactivity of each monoclonal antibody for the Clone A mutant form of α -catenin.

that the mutation was not likely to be in this region. In order to identify the region of the mutation, a series of monoclonal antibodies which map to different regions (Figure 3A) of the α -catenin protein were used to probe Western blots of the 80 kD mutant form. The α 7A11 antibody which maps toward the amino-terminus of the protein (aa 228-326) did not recognize the 80 kD mutant form of α -catenin, therefore the mutation was thought to lie within this region (Figure 3B).

PCR primers were designed that flanked the corresponding suspicious region of the α -catenin cDNA to map the exact mutation by RT-PCR. RT-PCR of Clone A mRNA using α -catenin primers A and D (Figure 4, panel A/D) produced the expected size fragment of 1058 bp but also a shorter fragment about 600 bp long, indicating a deletion of approximately 450 bp. Another PCR reaction with Clone A cDNA using the α -catenin primers B and D (Figure 4, panel B/D) produced a 350 bp fragment instead of the expected 804 bp fragment, confirming a deletion of approximately 450 bp 5' of the PCR primer D. All other primer combinations spanning the remainder of the α -catenin cDNA gave normal size PCR products

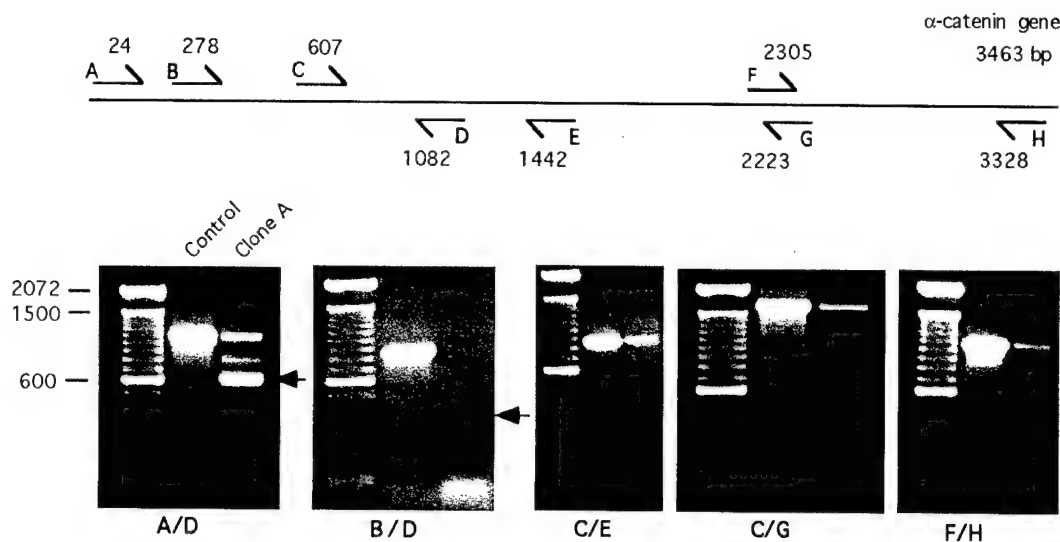


FIGURE 4 RT-PCR of Clone A mRNA using α-catenin primers A and D produced PCR fragments of the expected size in addition to smaller size fragments reflecting a deletion, as indicated by the arrow on the right. Primers B and D also produced smaller sized fragments than expected, confirming the same size deletion seen with the previous primer set. Primers C/E, C/G and F/H, which span the remainder of the α-catenin gene gave only expected size PCR products. The control in each case is the PCR product of a full length α-catenin cDNA.

(Figure 4, Panel C/E, C/G, F/H). The 600 bp PCR product resulting from amplification with primers A and D was cloned and sequenced revealing a 474 bp deletion from bp 590 to bp 1063 inclusive, in the α-catenin cDNA. This mutation deletes exons 4 and 5 of the gene (Furukawa, et al., 1994) which amounts to a loss of 158 aa from aa 197 to aa 354 of the α-catenin protein, a region with no homology to vinculin (Figure 5).

Immunolocalization of Components of the Adhesion Complex in Clone A cells.

Although Clone A cells do not exhibit the normal "epithelial" phenotype, E-Cadherin (Figure 6 panel B), β-catenin (Figure 6 panel D) and α-catenin (Figure 6 panel F) are all localized at the junctional membrane of cell-cell interactions. In addition, α-catenin antibodies stain an unidentified spindle-like structure that is not associated with the nucleus (Figure 7 panel A). This spindle-like structure is also apparent in Clone A cells stained for β-catenin

(Figure 7 panel B/white arrows), co-localization of β-catenin with the mutant α-catenin is not surprising since we've shown by co-immunoprecipitation that these two proteins can still bind to each other (Figure 10). Both catenins also appear to stain the nucleus in

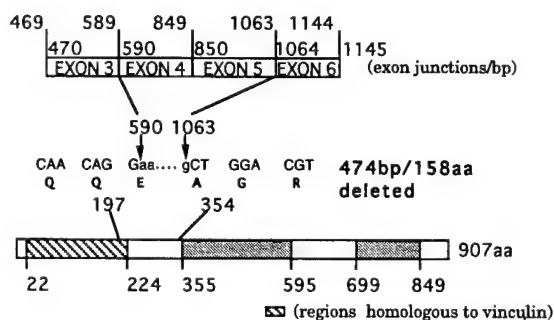


FIGURE 5 Schematic showing the region of the α-catenin deletion in Clone A cells and the regions of vinculin homology across the protein. The deletion removes 474 bp from base pair 590 through base pair 1063, of the α-catenin cDNA. This results in an in frame deletion of 158 aa of the α-catenin protein from aa 197-aa 354, a region with no homology to vinculin.

some Clone A cells (Figure 7 panel B/black arrow). However, we believe this represents nuclei overlying

the spindle-like structures, rather than true nuclear staining.

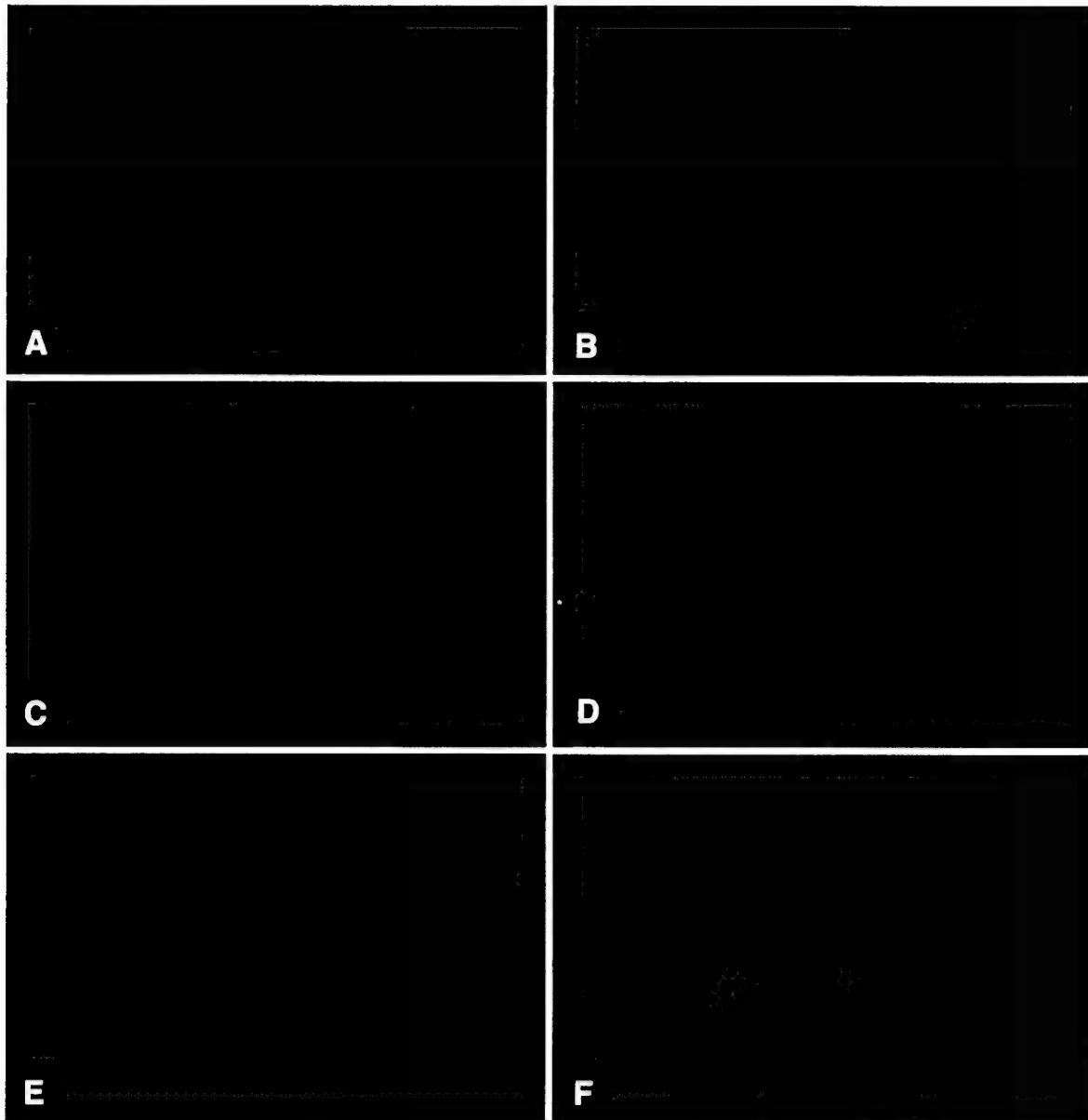


FIGURE 6 Immunolocalization of components of the adhesion complex in the non-adhesive Clone A cells is different than that seen in adhesive HT-29 cells. Clone A cells (panels B, D and F) were stained with various primary antibodies followed by a CY3 conjugated secondary antibody and compared to the equivalent staining of HT-29 (colon ca) controls (panels A, C, and E). Panels A and B are stained with E-cadherin (TLE1), C and D with β -catenin (YR6), and E and F with α -catenin (α 3H4). Although Clone A cells do not exhibit an "epithelial phenotype" both E-cadherin (panel B) and β -catenin (panel D) are localized at the membrane of cell-cell interactions. Alpha-catenin (panel F) shows a more unusual staining pattern, although, there is membrane staining most of the staining appears to be associated with an unidentified spindle-like structure. Panels A through F were all viewed and photographed at an original magnification of 400X. (See color plate I)

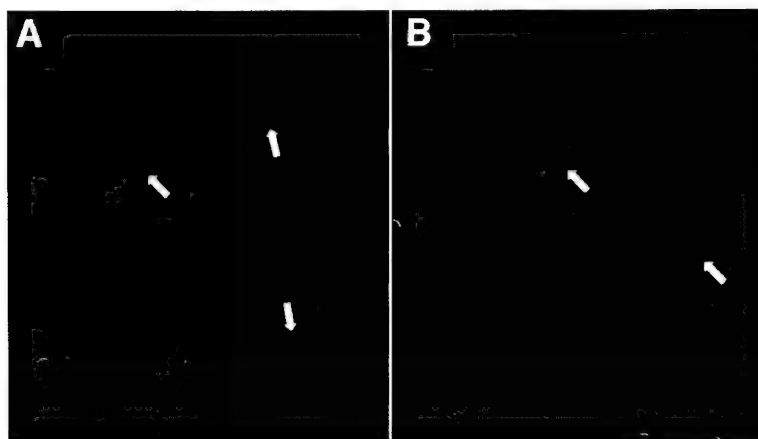


FIGURE 7 Immunostaining of alpha and beta-catenin in Clone A cells is associated with spindle-like structures. Clone A cells were stained with the α -catenin antibody α 3H4 (panel A) and the β -catenin antibody TL β 1 (panel B). Primary antibodies were detected by a CY3 conjugated secondary antibody (red). Nuclei were stained blue using DAPI (4',6-diamidino-2-phenylindole) contained in the mounting medium "Vectashield" (Vector Laboratories, Burlingame, CA). The white arrows indicate spindle-like structures not associated with the nucleus that are apparent in Clone A cells stained for both α -catenin (panel A) and β -catenin (panel B). The black arrow in panel B indicates β -catenin staining which might be confused for nuclear staining, but focusing in the Z plane suggests that it is a spindle-like structure beneath the nucleus. The bar in panel B is 20 μ m. (See color plate II)

Triton X-100 Solubility of Mutant α -Catenin

The detergent solubility of mutant α -catenin in confluent Clone A cells is similar to that of the full length protein in a control cell line (HT-29) (Figure 8). Since most of the mutant α -catenin remains triton

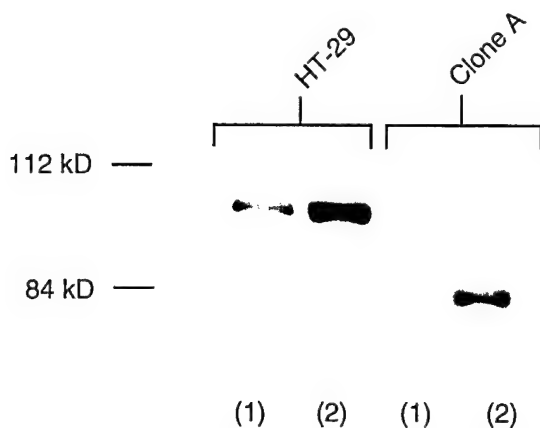


FIGURE 8 Triton X-100 solubility of mutant α -catenin in Clone A cells shows that the mutation does not alter the triton solubility of α -catenin compared to that of the HT-29 cell line. A Western blot is shown detected with a monoclonal antibody to α -catenin (TL α 1) of the triton soluble fraction (1) and triton insoluble fraction (2) of confluent Clone A cells compared to the HT-29 control. Most of the mutant α -catenin is associated with the triton insoluble fraction.

insoluble and by immunofluorescence we see a fraction of the total mutant at the plasma membrane (the remainder being associated with the spindle-like structures) we can speculate that the mutant α -catenin may be localized to a cytoskeletal or membrane component of the cell that is not associated with the plasma membrane.

Transfection of Clone A cells with Full Length α -catenin

Clone A cells grow as loosely adherent cells both to each other and to the extracellular matrix, they can be easily dispersed into a single cell population by very mild digestion with trypsin. As is characteristic of a poorly differentiated cell line Clone A cells are spindle shaped and fibroblastic in appearance and maintain very little contact with each other unless in a state of confluence. Forced expression of full length α -catenin in these cells induces a dramatic morphological change (Figure 9A). The cells become flattened, larger in size and they establish contacts with each other that are morphologically epithelial. Immunofluorescent staining shows that these morphological changes coincide with the expression of full length α -

catenin and its localization at the membrane (Figure 9B) as detected by the $\alpha 7A11$ antibody that does not recognize the mutant form. It is our understanding that the presence of normal levels of wild type α -catenin in Clone A cells re-establishes a functional E-cadherin-catenin complex at the plasma membrane, which rescues adhesion and restores the epithelial phenotype. Despite repeated attempts, we have been unable to isolate a homogeneous population of stably transfected cells. Most of the clones obtained exhibit a mosaic expression of the transfected α -catenin. They appear to be stable, but undergo growth arrest or

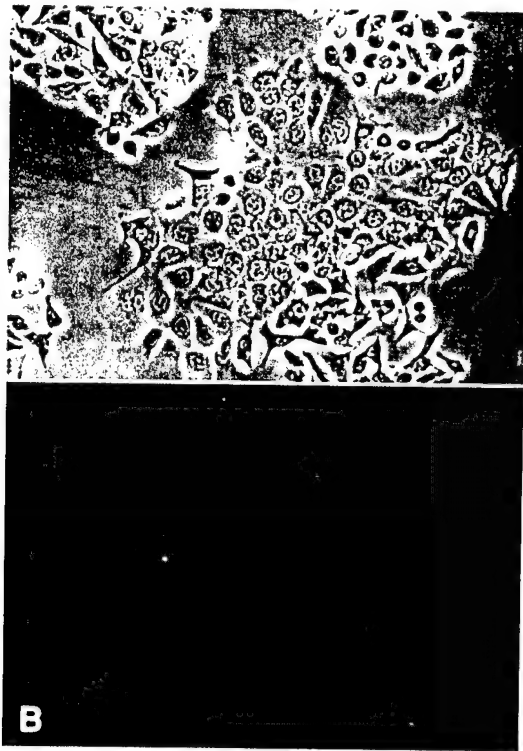


FIGURE 9 Transfection of Clone A cells with full length α -catenin shows rescue of the adhesive phenotype. Both panels show a mixed colony of Clone A cells transfected with the full length α -catenin cDNA. Panel A shows a phase contrast image of a small transfected colony showing a phenotype change where cells look larger and flattened and more "epithelial-like" than their non-transfected neighbours. Panel B shows the same field as panel A and is stained for the full length α -catenin (using monoclonal antibody $\alpha 7A11$, which does not recognize the endogenous mutant α -catenin). Strong membrane staining can be seen and is only associated with the transfected colony showing the phenotype change. Panels A and B were viewed and photographed at an original magnification of 400X. (See color plate III)

apoptosis, in that colonies can be propagated for months before they ultimately lose all expression.

Immunoprecipitation of Mutant α -catenin

To determine whether the mutant α -catenin could associate with various cytoplasmic proteins we conducted a series of immunoprecipitation experiments using Clone A whole cell lysates as shown in Figure 10. The mutant α -catenin found in Clone A cells can bind to β -catenin since immunoprecipitation of Clone A lysate with α -catenin, co-precipitates β -catenin as does the HT-29 positive control and can be seen on the blot as a band at approximately 96 kD (Figure 10A). The PC9 cell line was used as a negative control since it does not express any α -catenin protein (Oda, *et al.*, 1993; Shimoyama, *et al.*, 1992) and therefore would not be expected to co-precipitate β -catenin. The converse experiment has also been carried out where Clone A cell lysate was immunoprecipitated with β -catenin which co-precipitated the mutant α -catenin (result not shown). In addition to being able to bind β -catenin the mutant α -catenin can bind plakoglobin since immunoprecipitation with plakoglobin co-precipitates the mutant α -catenin (Figure 10B). Furthermore the mutant α -catenin is also able to form a complex indirectly with E-cadherin since immunoprecipitation with E-cadherin co-precipitates the mutant α -catenin (Figure 10B).

Interaction of Mutant α -catenin with F-actin

The ability of the mutant α -catenin to interact directly with F-actin was examined by conducting a cosedimentation experiment using the recombinant GST-Clone A α -catenin fusion protein and purified F-actin as shown in Figure 11. Both full length α -catenin and the mutant α -catenin alone, bind and bundle actin as evidenced by the presence of these proteins in the pellet fraction with actin. Beta-catenin alone does not bind actin as evidenced by the lack of both β -catenin and actin in the pellet fraction. A mixture of full length α -catenin with β -catenin does not cosediment in the absence of actin, neither does a mixture of the mutant α -catenin with β -catenin. Both the full length

α -catenin and the mutant α -catenin can bind β -catenin and cosediment actin at the same time as can be seen by the presence of all three proteins in the pellet fraction.

DISCUSSION

Although the Clone A line was originally described as being deficient in α -catenin (Breen, et al., 1993), we have multiple pieces of evidence to suggest that Clone

A cells possess both a mutant form and the full length α -catenin protein. Our evidence is fourfold. (1) RT-PCR of Clone A mRNA using primers A/D shows both mutant and full length size PCR products (Figure 4 Panel A/D). Although the full length size PCR product is not seen using primers B/D (Figure 4 panel B/D), our Western blot data suggests that the mutant form of the α -catenin protein is substantially more abundant in these cells. Presumably this correlates with a higher level of mutant message, which is preferentially amplified. (2) Northern blot analysis of

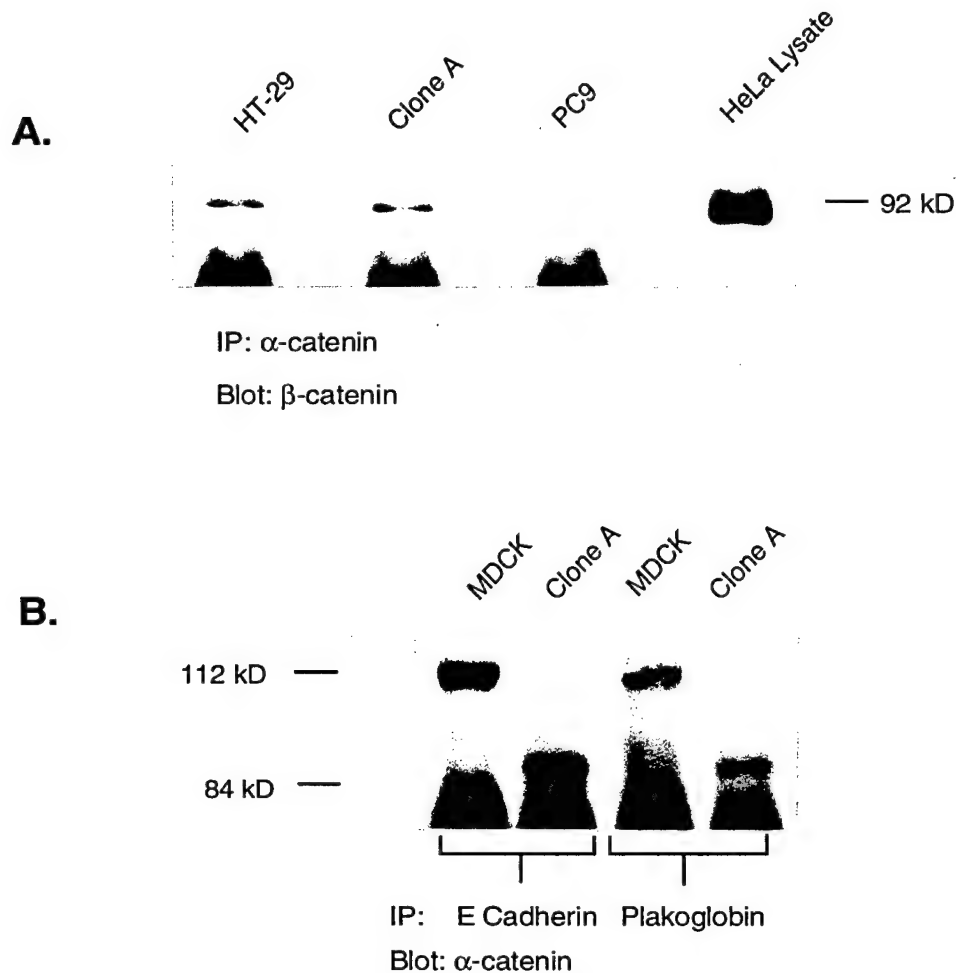


FIGURE 10 Immunoprecipitation and coprecipitation of α -catenin shows that the mutant form can bind to β -catenin and plakoglobin. Panel A shows samples that have been immunoprecipitated (IP) with a monoclonal antibody to α -catenin (TL α 1) and Western blotted (Blot) with a monoclonal antibody to β -catenin (TL β 1). Panel B shows samples that have been immunoprecipitated with monoclonal antibodies to E-cadherin (TLE1) and Plakoglobin (TLP1) and Western blotted with the monoclonal α -catenin antibody (TL α 1).

Clone A mRNA shows two different size messages (data not shown). (3) Western blots of Clone A cell lysates for α -catenin show the predominant mutant form but also small amounts of the full length protein (Figure 2). Figure 2 shows an overloaded Western blot designed to demonstrate that some full length protein is present. We estimate that the ratio between mutant and full length protein is between 100:1 and 1000:1. This may explain why the full length protein is not seen on routine Western blots and on other figures in this paper (eg Figure 8 and Figure 10B). (4) Immunofluorescent staining of Clone A cells with antibodies that recognize only the full length α -catenin protein (α 7A11) show weak but detectable staining at the plasma membrane under certain conditions (data not shown). These data raise the possibility that the α -catenin deletion in Clone A cells produces a phenotype which may be the result of a dosage effect of the preferentially expressed mutant.

Immunolocalization of the mutant α -catenin in Clone A cells shows staining associated with an unidentified spindle-like structure (Figure 6 panel F). This staining appears to be mutant-specific since it is not seen when using an α -catenin antibody that only recognizes the full-length protein and not the mutant form (Figure 9B). Although the mutant α -catenin is not entirely localized to the plasma membrane in Clone A cells it is clearly associated in some way with

a cytoskeletal fraction of the cell since the majority appears to be triton insoluble (Figure 8). We believe this may be due to connections between α -catenin and the cytoskeleton mediated by the intact actin binding site or perhaps through other intact cytoskeletal connections (possibly including α -actinin).

Despite the aberrant localization of the mutant α -catenin protein in Clone A cells our immunoprecipitation and coprecipitation data suggests that it can still interact with other known components of the adhesion complex although its affinity for these components may be abnormal. Our results show the mutant protein can bind E-cadherin (Figure 10B) via β -catenin (Figure 10A) and plakoglobin (Figure 10B) and could simultaneously cross link this complex to the cytoskeleton by binding to actin (Figure 11) in much the same way as the full length protein could. Thus, with the exception of the interaction with α -actinin, all currently published interactions of α -catenin with other proteins have been tested. Surprisingly, none of these interactions seem to be altered by the deletion mutation, even though the mutation abrogates adhesion. We conclude that α -catenin participates in other interactions in the cell, including other connections to the cortical cytoskeleton, that are required for functional adhesion. Although it is tempting to speculate that it may be a single

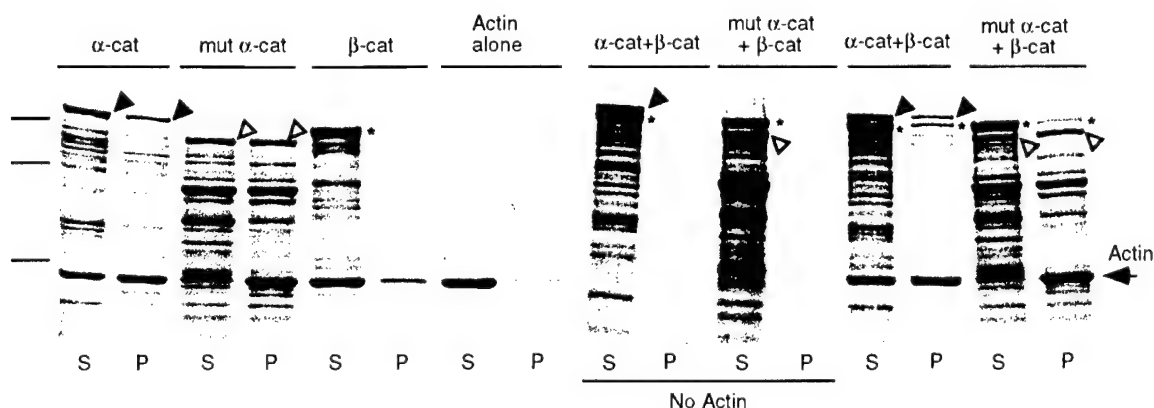


FIGURE 11 Cosedimentation of mutant α -catenin with F-Actin. Supernatant (S) and pellet fractions (P) of low speed (10,000 $\times g$) actin cosedimentation samples run on a 9% polyacrylamide SDS gel stained with Coomassie blue. Molecular weight markers of 97.4, 66.2 and 45 kD are shown on the left. Above each set of lanes are the components included in the sedimentation. Filled arrow heads indicate full length α -catenin, open arrow heads indicate the Clone A mutant α -catenin and the asterisk indicates β -Catenin.

interaction with a single cortical protein, we cannot exclude a multiple or complex interaction.

The concept of multiple connections to the cytoskeleton introduced by this mutation is attractive. Work of Nagafuchi and colleagues using cadherin- α -catenin chimeras showed that even though only chimeras with the C-terminal half of α -catenin could mediate adhesion, those with the N-terminal half were also triton insoluble (Nagafuchi, et al., 1994). The interaction of α -catenin with α -actinin (Knudsen, et al., 1995) and the recent mapping of the site of interaction to a region distinct from that which binds actin (Nieset, et al., 1996) provides another example of a potential second cytoskeletal interaction site. Finally, our own in vitro work showing an interaction between α -catenin and spectrin (Lombardo, et al., 1994) suggests another potential interaction with a cytoskeletal element. These findings are all consistent with our current result suggesting, for the first time, that multiple cytoskeletal interactions are required for adhesion. In future work, it will be important to explore the potential contributions of these interactions to the assembly and stabilization of the adhesion complex.

Acknowledgments

The authors thank Tom D'Aquila for excellent technical assistance and thank Dr. J.S. Morrow for discussions of the work and review of the manuscript. This work was supported in part by grants from the Patrick and Catherine Weldon Donaghue Medical Research Foundation and from the US Army Research and Material Command DAMD17-94-J-4366 (to D.L.R.).

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Expression of *c-met* Is a Strong Independent Prognostic Factor in Breast Carcinoma

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Presented as a poster at the United States and Canadian Academy of Pathology Meeting, Orlando, Florida, March 1-7, 1997.

Supported by grants from the William and Catherine Weldon Donaghue Foundation for Medical Research (D.L.R.) and the U.S. Army (DAMD17-94-J4366) (D.L.R. and E.R.F.).

This work is dedicated to patients with breast carcinoma and their families, including relatives of the authors, Ruthy and Robin Brown, Barbara Rimm, and Mary Ruth Reitz.

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Received October 24, 1997; accepted November 18, 1997.

BACKGROUND. The *c-met* protooncogene encodes the *met* protein, the receptor for scatter factor/hepatocyte growth factor, a growth factor that modulates the motility and stable interaction of the epithelial cells. This study assesses the expression of *met* receptor in breast carcinoma and its prognostic value with respect to survival.

METHODS. Immunofluorescence was used to evaluate 91 archival breast carcinoma specimens using a polyclonal antibody to the cytoplasmic domain of the receptor. Cases were scored by two pathologists on a percentage basis and then converted to binary scores (positive or negative) on the basis of a bimodal distribution.

RESULTS. Strong expression of *met* was found in 20 invasive ductal breast tumor specimens (22%). The 5-year survival of patients whose tumors showed decreased *met* expression was 89%, in contrast to a 52% 5-year survival rate in patients whose tumors expressed *met* ($P = 0.008$). This trend also was observed in patients without lymph node metastases at presentation, in whom *met* negative patients had a 95% 5-year survival compared with only 62% for *met* positive patients ($P = 0.006$). Multivariate analysis using the Cox proportional hazards model showed *met* expression to be an independent predictor of survival, with a predictive value nearly equivalent to that associated with lymph node status.

CONCLUSIONS. The authors conclude that expression of *met* in patients with invasive ductal carcinoma of the breast is a strong, independent predictor of decreased survival and may be a useful prognostic marker with which to identify a subset of patients with more aggressive disease. *Cancer* 1998;82:1513-20.

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KEYWORDS: adhesion, scatter factor, hepatocyte growth factor receptor, survival.

The process of tumor progression, from its inception to advanced terminal stages, results from a multistep process including activation of oncogenes and inactivation of tumor suppressor genes. Detection of mutation or amplification of tumor-related genes was believed to be a potentially valuable prognostic indicator to augment the more traditional prognosticators of tumor size, lymph node status, and histologic type or grade.^{1,2} Unfortunately, the predictive value of most of these new markers has been less than that achievable with the conventional standards. As a result, there is no useful standard for the prediction of recurrence in patients with no lymph node metastasis at presentation.

To have prognostic value, a new marker must have predictive value as strong as, and independent of, the current standards. The receptor for scatter factor/hepatocyte growth factor (SF/HGF), *met*, may be an example of such a marker. SF/HGF is a mesenchymal cell-derived protein that dissociates epithelial cell colonies by causing a breakdown of intercellular junctions, scattering contiguous sheets of cells, and appears to modulate cell motility and invasion.³⁻⁸ The *c-met* protooncogene product has been identified as SF/HGF receptor.⁹

The *met* receptor is expressed selectively in several normal human epithelial tissues as well as in carcinoma.¹⁰⁻¹² The *c-met* encoded receptor is a 190-kilodalton (kD) glycoprotein comprised of a transmembrane 145-kD β subunit and an extracellular 50-kD α subunit¹³ and is in the tyrosine kinase family of receptors.⁸ The ligand-activated cytoplasmic domain of the *met* receptor is responsible for transducing the signal for cell dissociation, motility, and mitogenesis³ and recently has been specifically associated with tubulogenesis.¹⁴

Consideration of SF/HGF as a prognostic marker initially was stimulated by the finding of immunoreactive HGF in tissue extracts from primary human breast carcinoma but not in human breast carcinoma cell lines.¹⁵ Investigation of the level of expression of HGF message¹⁶ and protein in breast carcinoma has been performed¹⁷ and has shown to be a significant independent factor in predicting survival.¹⁸ Because breast carcinoma cells respond to SF/HGF as a mitogen, they must express the *met* receptor. The expression of *met* in breast tissue¹⁹ and breast carcinoma has been demonstrated²⁰ and correlated with recurrence of the disease.²¹ Two groups have examined both HGF and *met* in benign and malignant breast tissue and shown that there frequently is coexpression in carcinoma.^{22,23} Furthermore, they found that both are overexpressed in breast tumors compared with benign lesions. Other sites also have been examined and it appears that *met* expression may have prognostic value in gastric carcinoma, in which expression correlates with higher stage and grade tumors²⁴ and in prostate carcinoma, in which it is expressed more frequently in metastatic tumors than primary lesions.²⁵

To evaluate the independent prognostic significance of expression of the *met* protein in breast carcinoma, we studied *met* expression in 91 breast carcinoma patients. In this study, we show the relation of *met* expression with age, tumor size, lymph node involvement, estrogen receptor (ER) status, and survival.

MATERIALS AND METHODS

Polyclonal Antibodies

The "*c-met*" polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) is affinity purified rabbit immunoglobulin G antibody against the synthetic peptide corresponding to the last 12 amino acids at the carboxyl terminal of human *met* protein.

Patient Population

The cohort included 91 patients who underwent breast resections at Yale-New Haven Hospital for invasive ductal breast carcinoma. Approximately 50% presented with metastases to regional lymph nodes. The

age at diagnosis ranged from 26–88 years (average, 58.1 years) and survival time ranged from 39 days to 14.1 years (average, 5.4 years; median, 5.1 years). The cohort showed an 88% 10-year survival of lymph node negative patients and a 45% 10-year survival of lymph node positive patients, which compares well with the literature²⁶ and suggests that this is a representative population. Clinicopathologic parameters collected on each case included age, tumor size, tumor histologic grade, ER status, lymph node involvement, and survival. Other parameters including progesterone receptor (PR), ploidy, and histologic and nuclear grade were collected, but these parameters were available for < 40 cases. To complete the analysis, nuclear grade and histologic grade were assigned retrospectively by a pathologist (D.L.R.) according to the method described by Fisher et al.²⁷ All material was collected under the auspices of the Critical Technologies Program at Yale and in accordance with human investigation committee protocol 8219 to the principal investigator (D.L.R.).

Immunostaining

Sections were obtained from 91 cases of invasive ductal breast carcinoma. Additional sections were derived from ten cases of benign breast tissue specimens for control. Standard histologic sections were cut from formalin fixed, paraffin embedded blocks and baked at 60 °C overnight. Sections were deparaffinized with xylene, rehydrated, and then blocked with a mixture of methanol and 30% hydrogen peroxide and prepared for immunostaining using a pressure cooker antigen retrieval method.²⁸ Overnight incubation was performed at 4 °C in a humidity tray with a 1:250 dilution of the polyclonal *c-met* primary antibody (Santa Cruz Biotechnology, Inc.) in 0.3% bovine serum albumin (BSA) in Tris-buffered saline (TBS). After incubation, slides were washed 7 times with TBS including 0.01% triton X-100 in the sixth wash. For increased sensitivity and better subcellular localization, Cy3-conjugated secondary antibodies were used rather than the conventional enzymatic reaction-based chromogens. Cy3-goat antirabbit antibody (Jackson ImmunoResearch Labs, West Grove, PA) was diluted 1:500 in TBS with 0.3% BSA and incubated for 1 hour before washing, as detailed earlier, and coverslipped. Slides were stored at –20 °C to maintain the fluorescent signal, which appears to be stable for > 1 year. Controls included no primary antibody and synthetic peptide (SC-10P; Santa Cruz Biotechnology) premixed with antibody at 1:1000 dilution (200 ng/mL) before incubation on the breast tissue.

Histologic Scoring and Analysis

Cases were examined on an Olympus AX-70 epifluorescence photomicroscope by two pathologists and

scored three times (twice by R.A.D.G. and once by D.L.R.). In each case, a serial hematoxylin and eosin stained section from the same block was examined for orientation and confirmation of the histologic diagnosis. Each case was scored blindly with respect to patient history, presentation, and previous scoring. Staining for *met* protein was evaluated in the tumor as well as in the normal breast, when present in the section (approximately 80% of the cases). A tumor sample was scored by the percentage of epithelial cells staining for *met* protein only if intense cytoplasmic and/or membranous reactivity was observed. No attempt was made to quantify intensity of staining. There was a clear, reproducible, and easily definable bimodal separation. Cases were either < 20–30% positive or > 70–80% positive. The ten cases of benign breast tissue were scored as controls and all were positive.

For analysis, we broke this study into two groups: a group that was positive for expression and a group that was negative for expression. Using this scoring system, there were no discrepancies in scoring between the two pathologists. Splitting of the negative cases into smaller groups was attempted, but no statistically significant differences were found and these smaller splits were less reproducible. In six lymph node positive cases, tumor also was examined in the lymph nodes. In each case the *met* expression score in the tumor in the lymph node was identical to that of the primary breast lesion.

Statistical Analysis

Patient follow-up information was obtained from Dr. Diane Fisher in conjunction with the Yale Comprehensive Cancer Center and the Connecticut Tumor Registry. The association of various disease parameters was analyzed by the chi-square test. The recurrence free survival and overall survival curves with or without lymph node involvement were generated by the Kaplan-Meier method. The Cox proportional hazards model was used to assess the independent prognostic value of each variable. All calculations were made using the StatView 4.5 Software on a PowerPC Mac 7100 (Apple Computer, Cupertino, CA).

RESULTS AND DISCUSSION

Previous studies have used this *c-met* primary antibody in immunostaining experiments, and it has demonstrated specificity for *met* protein.^{8, 29} In our own studies (Fig. 1A), the antibody recognizes a 145-kD band in lysates from breast carcinoma cell lines, with light bands at 190-kD and 170-kD (precursors). This is consistent with other descriptions of the mobility pattern by other investigators.¹⁰ Furthermore, we have shown that the staining pattern observed in represen-

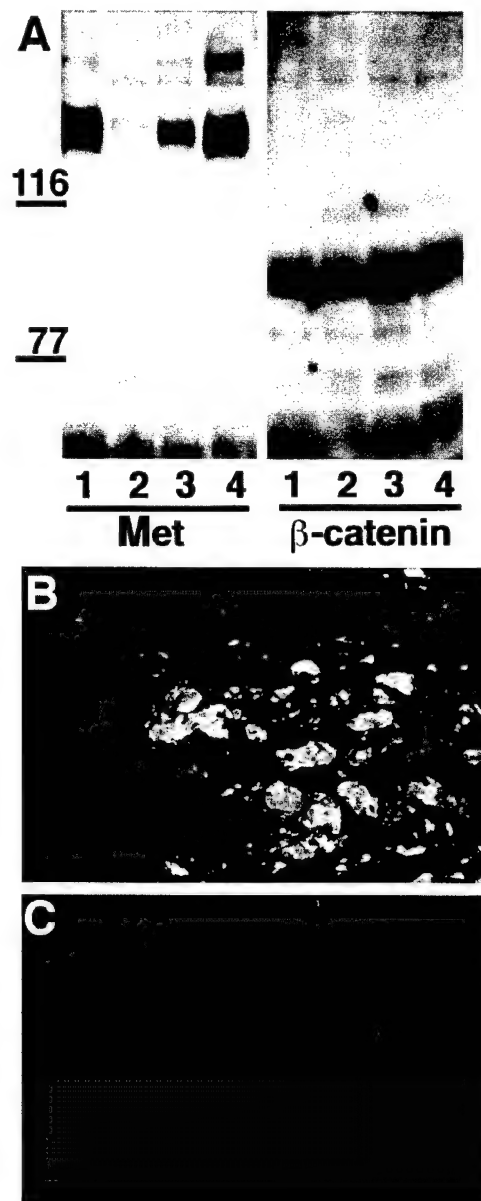


FIGURE 1. (A) Western blot analysis of four cell lines for *met* and β -catenin shows that the antibody used recognizes a 145-kilodalton (kD) band, as expected for the β subunit of *met*. The cell lines used were 1) A431, 2) MCF-7, 3) MB-468, and 4) HBL100. β -catenin, which migrates at 92 kD, is shown as a control for loading because the levels of expression of *met* vary in the cell lines chosen. (B) An example of the staining pattern observed with the "c-met" antibody visualized by Cy3 fluorescence is shown in a $\times 200$ magnification view of invasive ductal carcinoma invading adipose tissue. (C) A similar area in a serial section visualized after pretreatment with synthetic blocking peptide shows complete inhibition of staining of the invading carcinoma cells.

tative strongly reactive invasive carcinoma cases is completely inhibited by the addition of the synthetic peptide that was used for generation of the antibody (Figs. 1B and 1C).

In benign breast tissue cases studied (fibrocystic

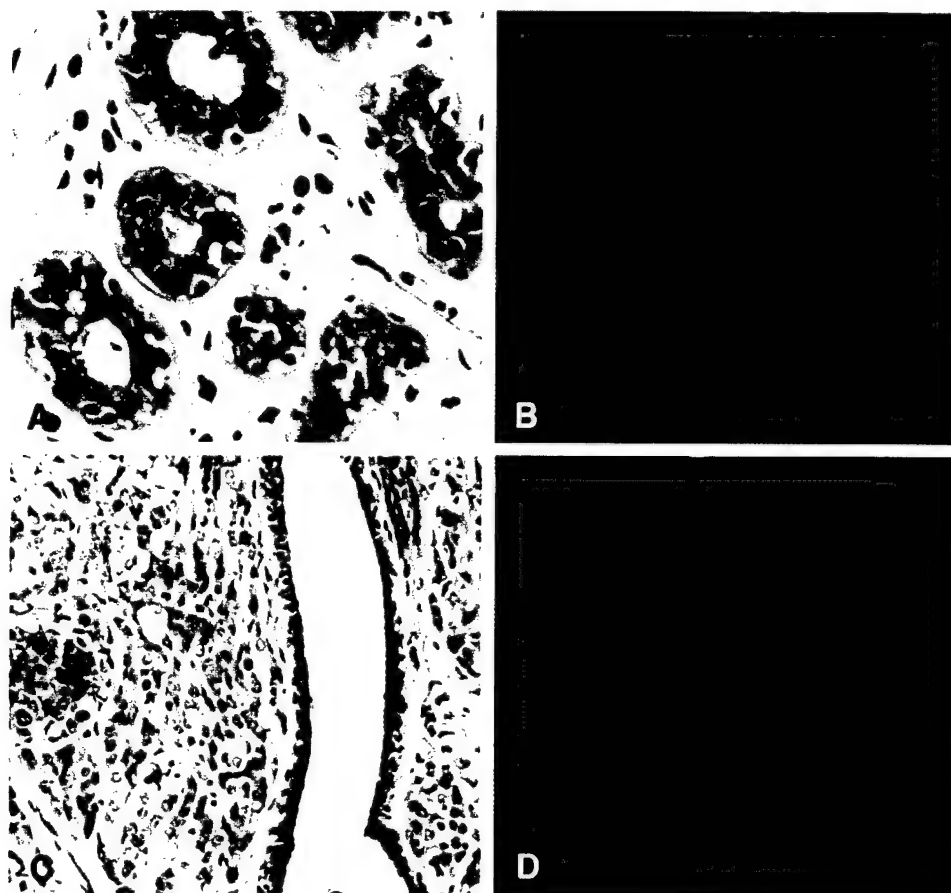


FIGURE 2. Sections with normal ducts and lobules show expression of *met* both at the membrane and diffusely, in the cytoplasm, but not in the nucleus. Figures A and C show hematoxylin and eosin stained images whereas Figures B and D show serial sections visualizing *met* reactivity with Cy3 fluorescent staining. The lobular units shown at $\times 400$ magnification in A and B show a diffuse cytoplasmic pattern. The larger duct shown at $\times 200$ magnification in C and D shows strong cytoplasmic staining and provides a good contrast to the near complete absence of staining of the tumor cells (visualized in the H & E image) surrounding the normal duct.

change, fibroadenoma, and normal ductal and lobular tissue) moderate to intense *met* staining in ductal and lobular epithelium was observed in all cases (Figs. 2A and 2B), with staining occurring essentially in all cells within a duct or lobular unit. Expression was present in the membrane and cytoplasm of the breast epithelium and absent in the stroma. Most cases of infiltrating ductal carcinoma showed marked loss of expression of *met*, with $< 5\%$ of cells having any immunoreactivity. Some cases had expression in 5–15% of the cells distributed randomly throughout the tumor. Nearly all cases, including many of those that were completely negative, had expression in adjacent normal ducts (Figs. 2C and 2D), serving as an internal positive control. Subdivision of cases by percentage of cells stained was examined and the distribution clearly was bimodal, so for purposes of scoring cases were divided into two groups, positive or negative. Examples of each illustrate typical negatives (Fig. 3A) with

$< 5\%$ of cells actually staining and typical positives (Fig. 3B) with nearly all ($> 90\%$) cells staining. No attempts were made to quantitate or analyze the intensity of staining or subcellular localization in this study.

Overall, 20 of the 91 invasive ductal breast carcinomas (22%) showed strong (positive) expression of the *met* protein. Distribution of the pattern of *met* expression in lymph node negative and lymph node positive patients is shown in Figure 4. Analysis was performed by the chi-square test to assess association between *met* positivity and lymph node status, age at presentation, hormone receptor status, and tumor size. No significant correlations were found except between the expression of *met* and tumor size ($P = 0.0348$) when a tumor size of ≥ 3 cm was chosen to separate the two groups.

The relationship between *met* expression in tumors and survival was assessed using the Kaplan-

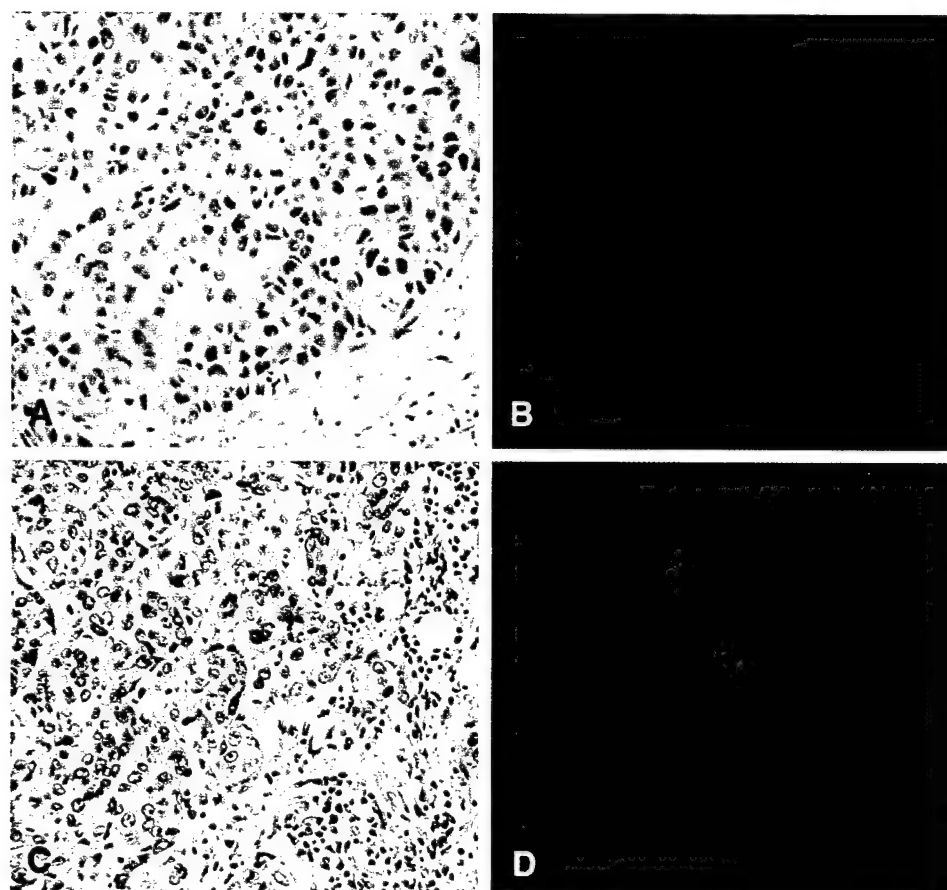


FIGURE 3. Illustration of cases of invasive ductal carcinoma with different *met* expression patterns. H & E (A and C) and immunofluorescent images (B and D) at $\times 200$ original magnification are shown as representative cases showing examples of a negative case (A and B) and a positive case (C and D) staining in regions of high cellularity in cases of infiltrating ductal carcinoma. Note this negative case shows some (20%) strongly staining cells and is an example of the highest staining levels still considered "negative." Figure 2D is a more typical negative case, with approximately 5% of cells staining for *met*.

Meier method. Significantly decreased survival was observed in patients expressing *met* (Fig. 5). In the *met* negative group, survival at 5 years was 89.6% and was 72.7% at 10 years, compared with 52% survival at both 5 and 10 years for the *met* positive group ($P = 0.008$ by Mantel-Cox log rank test) (Fig. 5A). When these cases were stratified by lymph node status at presentation, both lymph node positive and lymph node negative groups had survival curves suggesting worse prognosis in *met* positive tumors (Figs. 5B and 5C).

To determine the independent predictive value of *met* positivity and compare it with some established prognostic factors, the Cox proportional hazards model was used in both univariate and multivariate tests. The data, summarized in Tables 1 and 2, show that *met* has independent predictive value that is equal to or better than that of size or lymph node status and much better than age, nuclear grade, or histologic

grade. Note that for the multivariate analysis the ER variable was excluded because there were only 40 cases on which this value was determined. In a separate chi-square test, ER was not associated with *met* expression ($P = 0.99$). Because PR data were available for < 40 cases, no analysis was attempted.

Finally, we question whether the poor prognosis associated with *met* expression in tumors was a result of reactivation of *met* in some tumors or if expression was never lost. Data from cell lines suggest that the *c-met* gene might be expressed in normal tissues, lost in tumors, then regained as tumors become more aggressive.³⁰ To assess this, we were limited to only 6 cases in which a subsequent resection was performed at least 2–3 years after the primary resection scored in this study. We found two of six negative cases that converted to a positive phenotype in latter specimens. These data are insufficient to draw any conclusions, but at least they are consistent with cell line data that

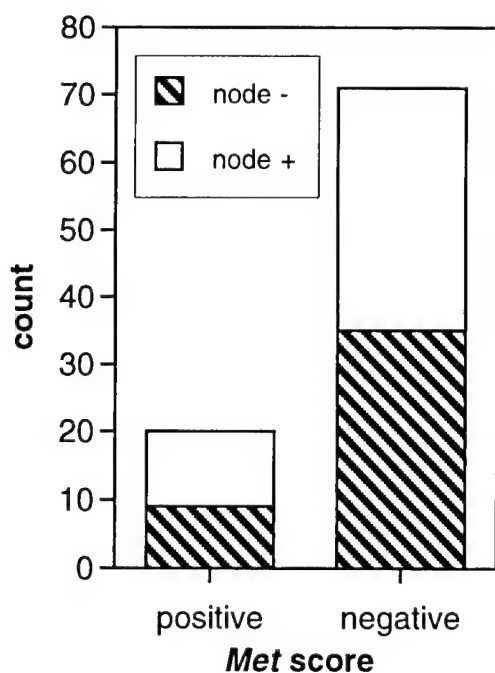


FIGURE 4. A frequency distribution of *met* expression in 91 cases of infiltrating ductal carcinoma of the breast split by *met* scoring and stratified by lymph node status at presentation. +: positive; -: negative.

suggest that *met* expression initially may be lost and then reactivated as tumors progress. If this proves to be true, it is possible that the association between *met* expression and poor survival may be even stronger than that estimated by this study because malignant cells were assessed at only one timepoint.

The possible reactivation of expression of *met* makes it an unusual marker. Other breast carcinoma markers have been observed being retained, as is the case for steroid receptors,^{31,32} or amplified, as is the case for HER-2/*neu*.^{33,34} Other phenotypes include increased protein expression observed for epidermal growth factor receptor³⁵ and p53³⁶ or decreased protein expression, as is the case for adhesion proteins.³⁷⁻³⁹ Although further study is required to quantitate expression levels, *met* expression may be unique in that it appears to be associated with tumor progression, being present in benign lesions, lost in invasive disease, and then reactivated or even overexpressed²² in a set of tumors with a poor prognosis, inferring a more progressive or advanced phenotype.

Because *met* is expressed in normal tissue,⁵ it is somewhat unexpected that its expression in tumors is strongly associated with poor prognosis. Conversely, activation of the *met* receptor in tissue culture cells by interaction with scatter factor results in dissociation of monolayers and a "scattering" of the cells, giving the epithelial cells a mesenchymal phenotype.^{3,40} Activation of *met* also activates expression of urokinase

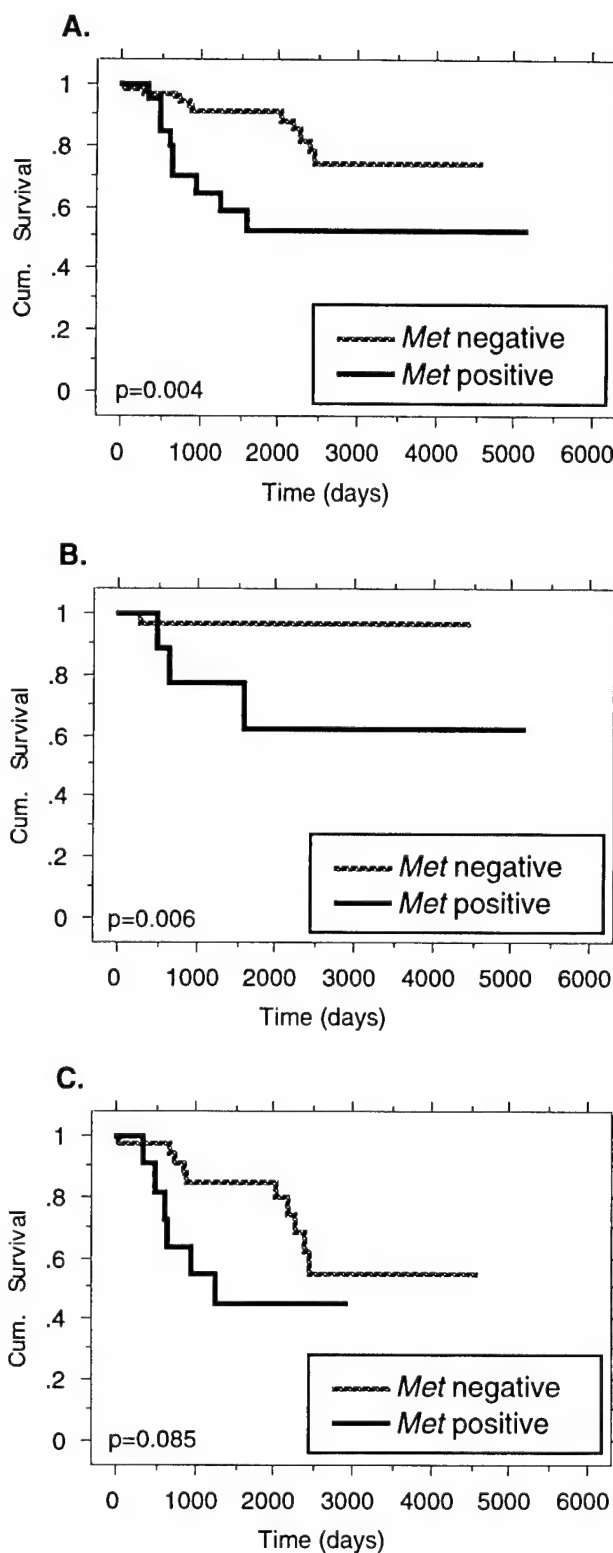


FIGURE 5. Kaplan-Meier survival analysis of cases by *met* expression, overall (A) and split by lymph node status at presentation in which (B) represents lymph node negative patients and (C) represents lymph node positive patients. Inset in the lower left corner of each plot is the *P* value testing the significance of the difference in the survival curves by the Mantel-Cox log rank test.

TABLE 1
Univariate Proportional Hazards Model

Variable	Relative risk	95% confidence interval	P value	No.
Met positive	3.34	1.38–8.06	0.007	91
Lymph node positive	4.25	1.42–12.6	0.010	91
Age <50 yrs	1.68	0.69–4.00	0.248	91
Estrogen receptor negative	1.84	0.41–8.28	0.427	40
Tumor >3 cm	3.46	1.25–9.61	0.017	90
Nuclear grade of 3	1.97	0.79–4.99	0.144	88
Histologic grade of 3	2.26	0.92–5.56	0.076	88

TABLE 2
Multivariate Proportional Hazards Model^a

Variable	Relative risk	95% confidence interval	P value
Met positive	3.47	1.22–9.90	0.020
Lymph node positive	4.09	1.19–14.1	0.025
Age <50 yrs	1.91	0.74–4.90	0.177
Tumor >3 cm	1.99	0.65–6.09	0.229
Nuclear grade of 3	1.46	0.38–5.48	0.577
Histologic grade of 3	2.37	0.66–8.43	0.183

^a Estrogen receptor is omitted because data are available for only 40 cases. For this analysis, n = 88 because size data were unavailable on 3 of the 91 c-met scored cases.

and its receptor, enabling cells to degrade extracellular matrix.⁴¹ These properties, which are consistent with expression in high grade malignancy, are less congruent with expression in a normal cell. Multiple functions have been attributed to *met*, depending on its ligand, context of expression, presence of extracellular matrix, and possibly other factors as well.⁴ Furthermore, recent studies suggest that the level of expression may be important. Jin et al. showed that overexpression of both *met* and scatter factor occurs in breast tumors when compared with normal tissue.²² Although we did not attempt quantitation in this study, we also observed that when *met* was expressed in tumor cells, they generally were brighter than adjacent benign ducts.

A large number of potential prognostic markers for breast carcinoma have appeared in the literature,^{42,43} but very few are proven. Typically markers may appear promising in early studies, but when rigorous statistical methods are applied, markers prove either ineffective or dependent on other known prognostic markers. Thus, relatively few standard markers including size, histologic type, nuclear and histologic grade, lymph node status, and ER/PR status have evolved as the recommended markers.^{26,44} In that light,

we present this study as a promising first study that can claim independent prognostic value in testing by the Cox proportional hazards model and has predictive value with respect to survival, even in lymph node negative patients. This work is supported by two previous studies addressing *c-met* and survival, one showing independent predictive value related to HGF/SF levels¹⁸ and another showing that loss of heterozygosity at the *c-met* locus is not associated with traditional markers but is predictive of decreased survival.⁴⁵ Furthermore, the simplicity of the technique and the general availability of the required reagents makes this any easy assay for any pathology laboratory. Further studies are underway, with emphasis on lymph node negative patients, to evaluate the prognostic significance of *c-met* expression.

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The Expression of p120ctn Protein in Breast Cancer Is Independent of α - and β -Catenin and E-Cadherin

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Several studies have reported loss or alteration of expression of E-cadherin in breast cancer and more recently changes in levels of expression of the catenins. We used immunofluorescence to examine E-cadherin, α -catenin, β -catenin, and p120ctn (formerly p120CAS) expression in 91 cases of invasive ductal carcinoma. As expected, all four proteins co-localize to the junctional regions of the cells. Although nuclear localization has been described for β -catenin in colonic polyps, no examples were found in these breast cancer cases. We found that, although alteration is common in the catenins and E-cadherin, complete loss, as exemplified by E-cadherin in lobular carcinoma (where E-cadherin is frequently mutated), is rarely seen. In contrast, the catenin-related protein p120ctn shows an expression pattern that is significantly unrelated to the other catenins (or E-cadherin), including complete loss of expression in approximately 10% of the cases. No statistically significant correlations with traditional prognostic indicators were observed with any of these proteins. We conclude 1) that expression of E-cadherin and α - and β -catenin are generally retained at the membrane although frequently reduced or altered, 2) that complete loss of p120ctn expression is seen in approximately 10% of the cases, and 3) that there is a significant correlation in the expression of E-cadherin and the catenins but no correlation between these molecules and p120ctn, suggesting an absence of coordinate regulation. (*Am J Pathol* 1998, 152:75–82)

Many studies have been done examining levels of expression of adhesion-associated markers with the goal of identifying an association with metastasis. Epithelial cell-cell adhesion is primarily mediated by E-cadherin (see Ref. 1 for review) and its associated cytoplasmic proteins, the catenins (see Ref. 2 for review). E-cadherin expression is frequently altered in both ductal and lobular carcinomas^{3–7} and E-cadherin gene mutations have been detected in lobular carcinoma.^{8–10} Alterations in

expression of α -catenin^{11,12} and also β -catenin and plakoglobin^{7,13} have been reported in breast cancer and breast cancer cell lines.¹⁴ Although no correlation with survival has yet been shown for the catenins, one study has recently suggested that E-cadherin alterations may be correlated with decreased disease-free survival.⁶ Other studies have shown no correlation.^{15–17}

Expression of the catenin-related protein p120ctn (formerly p120CAS) has not yet been examined in human breast tissue. This protein was first discovered as a major substrate of the src tyrosine kinase¹⁸ and several receptor tyrosine kinases, including the epidermal growth factor, colony-stimulating factor-1, and platelet-derived growth factor receptors.^{19,20} More recently, p120ctn has been defined as a member of the cadherin-based cell-cell adhesion complex.^{21–23} p120ctn contains a series of 42 amino acid *armadillo* repeats, placing it in the *arm* family with the other cadherin-associated proteins β -catenin and plakoglobin.²⁴ Additional studies showed p120ctn binds directly to E-cadherin, but unlike plakoglobin and β -catenin, it does not bind to either α -catenin or the adenomatous polyposis coli protein.²⁵ Biochemical studies suggest that p120ctn interacts with E-cadherin at a different site from the β -catenin/plakoglobin binding site.²¹ In addition, p120ctn co-precipitates with other members of the classical cadherin family (such as N- and P-cadherin), suggesting that the interaction is broadly applicable to cadherin biology. Although its exact function is still unknown, it may play a role in modulation of adhesion as the association of the tyrosine-phosphorylated p120ctn with E-cadherin is elevated in *ras*-transformed breast epithelial cell lines.²⁶

As p120ctn plays a role in modulation of adhesion, it may be another candidate for assessment of the metastatic potential of tumors, as down-regulation of adhesion is a primary event in metastasis.^{27,28} Unlike other adhesion proteins where there is loss of expression in tumor cell lines,^{14,29} p120ctn shows heterogeneous expression patterns, with isoform variability but, as yet, no evidence of complete loss of expression.³⁰ Several murine isoforms of p120ctn have been defined,³⁰ and at least six

Supported by grants from the William and Catherine Weldon Donaghue Foundation for Medical Research and the U.S. Army (DAMD17-94-J4366) to D. L. Rimm and E. R. Fearon and NIH grant (CA-55724) to A. B. Reynolds.

Accepted for publication October 24, 1997.

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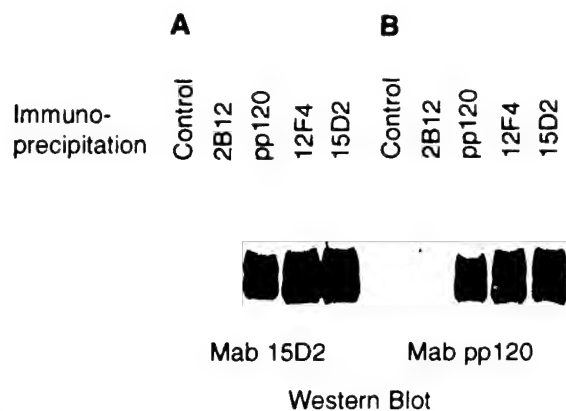


Figure 1. Specificity of p120ctn-specific MAbs. MDCK cell lysates were immunoprecipitated using the control MAb 12CA5 (control) or the p120ctn-specific MAbs listed across the top. Immunoprecipitates were separated on 8% polyacrylamide gels and then Western blotted with either Mab 15D2 (A) or Mab pp120 (Transduction Labs; B).

human isoforms are currently in the process of characterization (Frans Van Roy, personal communication). This isoform diversity is more complex than other cadherin-associated proteins of the adhesion complex, but alternative splice forms have been described for both β -catenin³¹ and α -catenin.³²

In this study we compared expression of p120ctn to E-cadherin and the conventional catenins. Tissue sections were labeled with Cy3-conjugated fluorescent antibodies rather than conventional enzyme-conjugated techniques to increase the signal-to-noise ratio to the

point at which loss can be reliably distinguished from alteration, or decreased intensity, as is frequently seen for these adhesion-related proteins. To calibrate our assay, we use 10 cases of lobular carcinoma as a reference for true loss of E-cadherin expression. Confirming the more recent literature, we found alteration of E-cadherin and α - and β -catenin common but true loss extremely rare. This pattern is different from that seen for p120ctn where there is true loss in approximately 10% of the cases (as defined by loss of reactivity of multiple monoclonal antibodies). Furthermore, we find co-localization but no correlation in level of expression of p120ctn and the other adhesion proteins examined, suggesting independent regulation.

Materials and Methods

Tissue Acquisition and Study Population

The cohort included 91 patients who have had breast resections at Yale-New Haven Hospital for invasive ductal breast cancer. Approximately one-half of them presented with metastases to regional lymph nodes. The age at diagnosis ranged between 26 and 88 (average, 58.1) years, and survival time ranged from 39 days to 14.1 years (average, 5.4 years; median, 5.1 years). The cohort showed an 88% 10-year survival of node-negative cases and 45% 10-year survival of node-positive cases, which compares well with the literature³³ and suggests that this

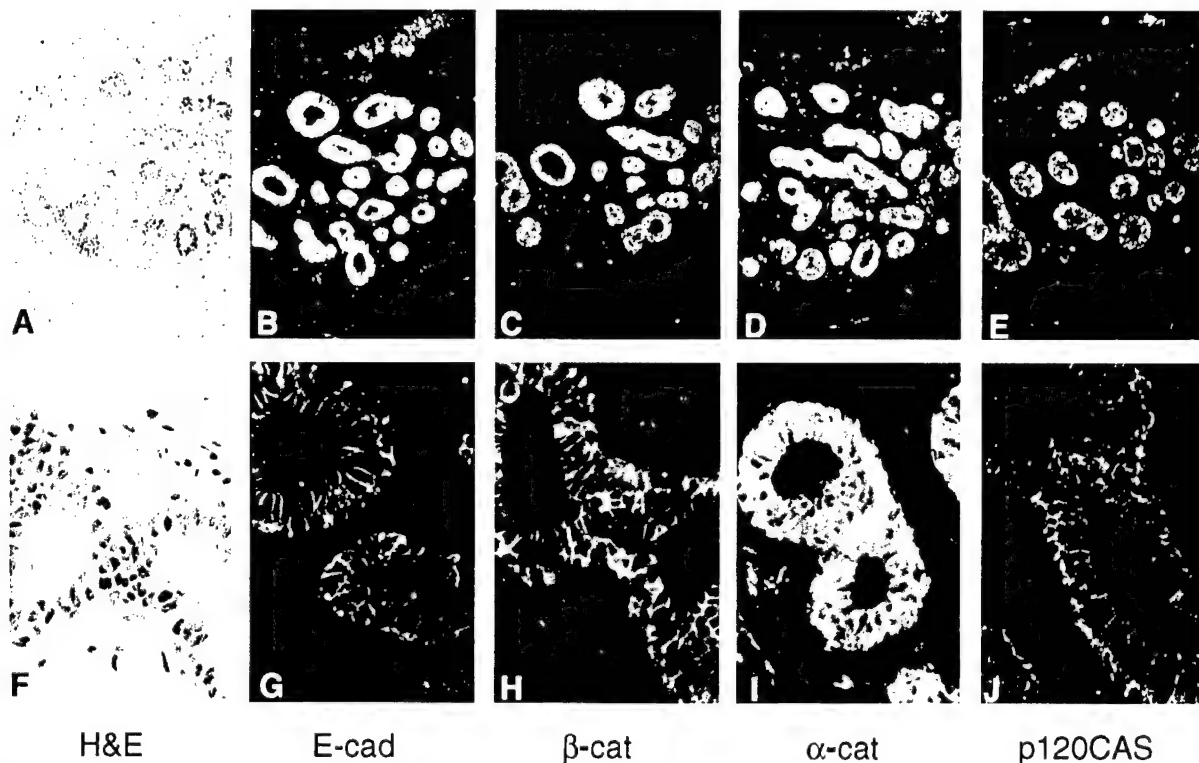


Figure 2. Normal staining patterns of all four proteins. The adhesion proteins E-cadherin, α -catenin, and β -catenin show classic basolateral membrane staining in normal breast ducts and lobules. As described in other tissues, the localization of p120ctn is essentially indistinguishable from E-cadherin and the catenins. Stains are as described in Materials and Methods and shown at low ($\times 20$ original magnification A to E) and high ($\times 80$; F to J) power. Stains, shown beneath each column, are H&E (A and F), E-cadherin (B and G), β -catenin (C and H), α -catenin (D and I), and p120ctn (E and J).

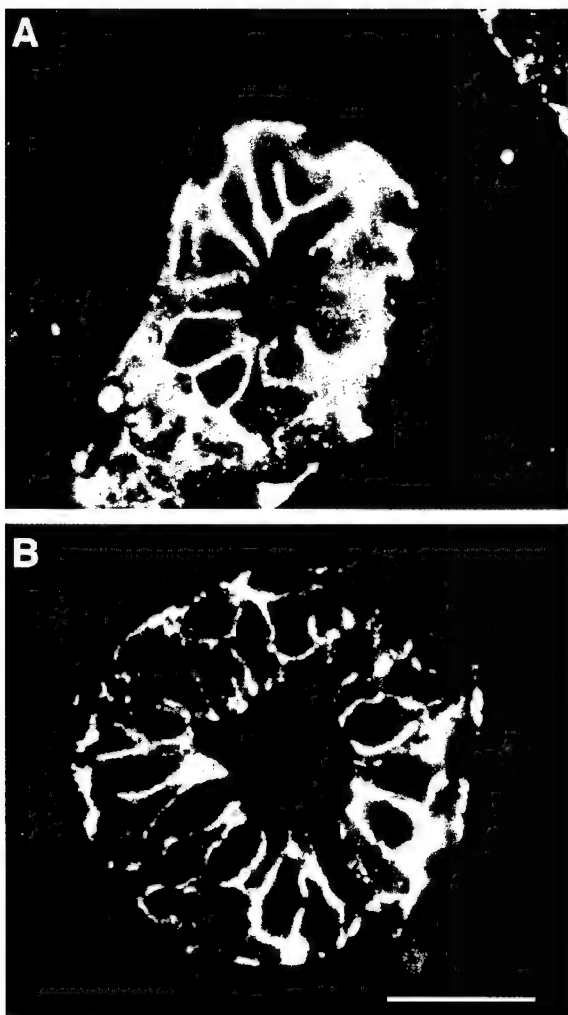


Figure 3. The subcellular localization of p120ctn is very similar to that seen for E-cadherin. High magnification views ($\times 100$ original magnification) shows bright membranous staining on the basolateral surfaces of the epithelial cells lining the small ducts for both E-cadherin (A) and p120ctn (B). Scale bar in B, 20 μ m.

is a representative population. Clinicopathological parameters collected on each case included age, tumor size, tumor histological grade, estrogen and progesterone receptor status, lymph node involvement, and survival. This population is ethnically and racially diverse although, due to the nature of the disease, includes only females. All material was collected under the auspices of the Critical Technologies Program at Yale and in accordance with human investigation committee protocol 8219 to the principal investigator (D. L. Rimm). Ten cases of lobular carcinoma were also selected at random to use as controls for loss of cadherin staining. No clinical information was obtained on this group.

Antibody Preparation

Recombinant fusion proteins were prepared from full-length human cDNA clones for both α -catenin and β -catenin by expression in glutathione-S-transferase (GST)-based expression vectors (Pharmacia, Piscata-

way, NJ). Each was purified on a glutathione affinity matrix, and antisera were raised in rabbits by injection in complete Freund's adjuvant. Antibodies were affinity purified in two steps. Anti-GST activity was depleted by passage over a column of Affi-gel linked to GST. The eluent was subsequently passed over an Affi-gel column with bound α -catenin or β -catenin. After washing, antibodies were eluted with 100 mmol/L glycine/HCl, pH 2.5. Fractions containing active antibodies as detected by enzyme-linked immunosorbent assay (ELISA) were pooled, dialyzed into phosphate-buffered saline containing 1 mmol/L sodium azide, and stored at -20°C . A commercial monoclonal antibody (MAb) to E-cadherin was used (Transduction Laboratories, Lexington, KY).

The p120ctn-specific MABs 12F4 and 15D2 were chosen for their excellent reactivity with formaldehyde-fixed tissue sections from a panel of p120ctn-specific MABs prepared previously (Wu and Reynolds, manuscript in preparation). These antibodies bind to different epitopes in the carboxyl-terminal 121 amino acids of p120ctn and recognize all known isoforms of p120ctn that can be distinguished by immunoprecipitation and Western blotting analysis. Both MABs were affinity purified on protein-A Sepharose columns. For immunostaining experiments, MABs 12F4 and 15D2 were used at 7 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$, respectively. Other p120ctn MABs, including 9D5, 5A7, 6H11, 8D11, 9B8 (Wu and Reynolds, manuscript in preparation), and pp120 (Transduction Laboratories), were used on some cases for confirmation of the staining pattern.

Immunostaining

Standard histological sections were cut from paraffin blocks and prepared for immunostaining using a pressure cooker antigen retrieval method.³⁴ Each section was baked at 60°C overnight and then deparaffinized and treated for antigen retrieval by immersion in 6.5 mmol/L sodium citrate (pH 6.0) for 5 minutes in a conventional pressure cooker (KMart). Sections were then blocked with 0.3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) (150 mmol/L NaCl, 20 mmol/L Tris, pH 8). MABs were diluted to 2 to 7 $\mu\text{g/ml}$ and incubated in a humidity chamber overnight before washing seven times with TBS including 0.01% Triton X-100 in the sixth wash. For signal-to-noise ratio and better subcellular localization, Cy3-conjugated second antibodies were used instead of the conventional enzymatic reaction-based chromogens. Cy3-goat anti-mouse or rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was diluted 1:500 in TBS with 0.3% BSA and placed in the sections for 1 hour before washing as above and coverslipping. Slides were stored at -20°C to maintain the fluorescent signal, which appears to be stable for over 1 year under these conditions.

Histological scoring and analysis

Each slide was examined on at least two separate occasions by at least two individuals including two patholo-

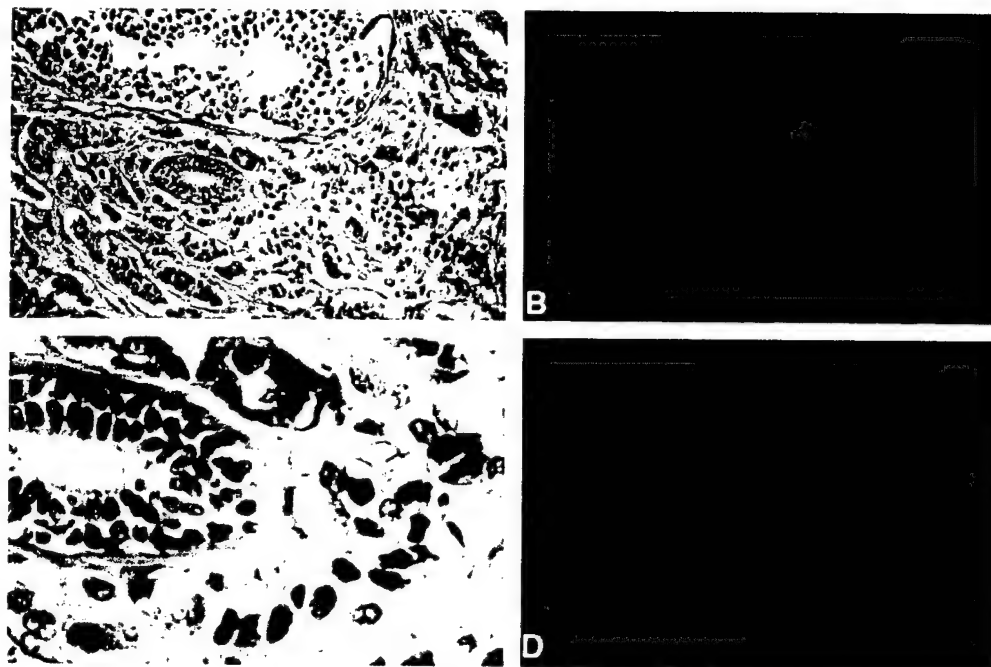


Figure 4. Example of altered staining of α -catenin in the tumor surrounding a normal duct. H&E stains (A and C) at $\times 20$ and $\times 80$, respectively, show a normal duct surrounded by a poorly differentiated ductal carcinoma. The staining pattern showing α -catenin expression (B and D) at similar magnifications shows normal staining surrounded by a pattern designated altered but not lost.

gists and a technologist using either a Zeiss epifluorescence microscope or an Olympus AX-70 epifluorescence photomicroscope. The expression of each antigen was scored as one of three final groupings. The final grouping used were 1) normal, cases not reproducibly distinguishable from normal, in either pattern or intensity; 2) altered, a broken or discontinuous staining pattern, or a patchy staining pattern, with or without a decrease in intensity; and 3) loss, a complete loss of staining as the predominant pattern in the section examined, comparable to that seen for E-cadherin in cases of lobular carcinoma, where E-cadherin is frequently mutated. In each case, a serial hematoxylin and eosin (H&E) section was examined for orientation and confirmation of the histological diagnosis. Each case was scored blindly with respect to patient history, presentation, and previous scoring. Patient follow-up information was obtained from Dr. Diane Fisher in conjunction with the Yale Comprehensive Cancer Center and the Connecticut Tumor Registry. Data analysis was done using StatView 4.5 for Macintosh.

Results

The specificities of p120ctn MAb 12F4 and 15D2 were compared by immunoprecipitation and Western blotting (from MDCK cell lysates) with the previously characterized MAb 2B12 and pp120 (from Transduction Laboratories) (Figure 1). MAb 2B12 specifically recognized the so-called CAS1 isoforms due to the location of its epitope in the amino-terminal 100 amino acids of p120ctn.^{21,30} This amino-terminal sequence is spliced out in many cell types, resulting in the faster migrating CAS2 isoforms, which are more abundant than CAS1 isoforms in this cell type. Both CAS1 and CAS2 isoforms were recognized by

MAbs 12F4 and 15D2, and their staining patterns were similar to those of MAb pp120. In addition to the similar immunoprecipitation and Western blotting patterns illustrated here, the properties of MAb 15D2 are nearly identical to that of MAb pp120 in every parameter tested to date, including immunofluorescence, species cross-reactivity, and isoform specificity. MAb 12F4 was chosen for the bulk of the experiments as it showed the best signal-to-noise characteristics in formaldehyde-fixed tissues. A selection of 30 cases were examined with 15D2 to confirm the staining pattern. Cases showing true loss of expression were re-examined with multiple MABs to confirm the loss of expression.

The adhesion protein p120ctn is associated with E-cadherin and shows some homology to β -catenin, but its role in stabilization or regulation of adhesion has yet to be defined. Like the catenins and cadherins, the protein localizes to lateral membranes. In breast tissue, it co-localizes exactly with E-cadherin and α - and β -catenin (Figure 2). At the resolution of immunofluorescence, the distribution of expression is predominantly basolateral and membranous with pale cytoplasmic expression (Figure 3). In some ductal cells there are increased membrane densities near the apex, presumably correlating with the location of the adherens junction. This finding is more prominent in colonic epithelium than in breast tissue.

Alteration of adhesion molecules in breast cancer has been described frequently in the literature.³⁵ Alteration or discontinuity of staining is grouped with loss in some studies, but that grouping may overlook a biological distinction. We have observed numerous cases where there are discontinuous or altered staining patterns, frequently with reduced intensity of adhesion protein staining, but

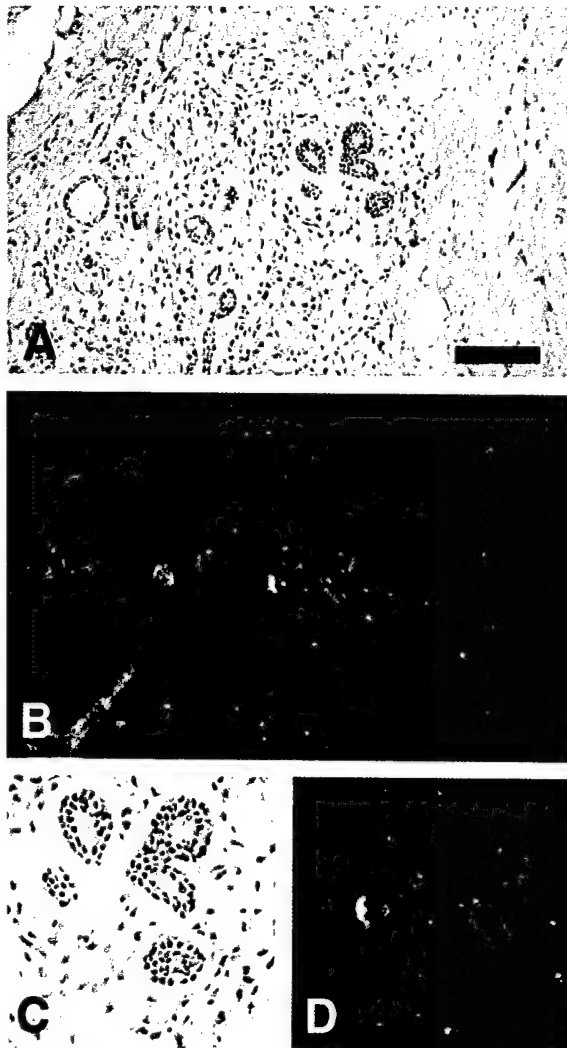


Figure 5. E-cadherin staining pattern in lobular carcinoma as an example of true loss of expression. Matched serial sections stained with H&E (A and C) and anti-E-cadherin visualized with Cy3-conjugated secondary antibodies (B and D) show low- and high-magnification views of normal small ducts surrounded by malignant cells of a lobular carcinoma. The immunofluorescent frames (B and D) are overexposed to show shadows of the malignant cells with complete absence of E-cadherin expression. Scale bar in A, 100 μ m.

true loss is rare. In this study, we define a category called altered for all cases with a staining pattern that is reproducibly and easily distinguished from normal but does not meet the criteria for true loss of expression. Generally, normal ducts are present in these cases and provide excellent internal calibration by which to make the judgment of alteration or loss. An example of alteration is shown in Figure 4, where tumor surrounds a normal duct. The staining pattern, defined as altered, shows broken and discontinuous but predominantly membranous staining. In this case the discontinuous α -catenin staining pattern is contrasted with the normal staining in the adjacent duct. The intensity may be reduced compared with normal ducts but is still easily seen. No attempt was made to quantify intensity.

True loss of expression is defined as a complete absence of antibody reactivity, similar to that seen in the

absence of the primary antibody. As a reference for this pattern we examined expression of E-cadherin in 10 lobular carcinoma specimens as mutations in E-cadherin occur in greater than 50% of the cases.⁹ We found 7 of 10 selected cases showed complete loss of expression of E-cadherin, as illustrated in Figure 5. Again, in each case enveloped normal ducts were used as an internal reference for normal antibody reactivity. Complete loss in the lobular carcinoma cells shows a staining pattern and intensity indistinguishable from the negative control.

Using these criteria for expression, we examined over 100 cases of ductal carcinoma of the breast. Each was scored on the basis of the consensus of at least two individuals. The distribution of expression pattern is shown in Figure 6. It is notable that complete loss of expression was never seen for α - or β -catenin and seen in only one case for E-cadherin. All cases showed membranous localization. Nuclear staining of β -catenin as reported in colonic polyps³⁶ was not seen in any case. In contrast, approximately 10% of the cases showed loss of p120ctn. To examine correlation of level of expression between proteins as a first assessment of coordinate regulation, the cases were analyzed using the χ^2 test. We found highly significant correlations between E-cadherin and α - and β -catenin but no correlation between p120ctn expression level and any of these adhesion proteins (Table 1); that is, frequently cases with normal E-cadherin and catenins showed altered p120ctn or vice versa.

The lack of correlation between p120ctn expression levels and the other proteins led to re-examination of these cases. True loss, as seen in 10% of the cases, was similar to loss of E-cadherin expression observed in lobular carcinoma. Normal-staining benign ducts were seen surrounded by nests of tumor cells with complete absence of p120ctn reactivity (Figure 7). These cases were recut for examination with other MAbs to confirm loss. The MAbs, including 5A7, 6H11, 8D11, and 9B8, showed a pattern of loss indistinguishable from 12F4. The 15D2 antibody showed a pattern of high background, with cytoplasmic staining but no true membranous staining as seen in the adjacent non-neoplastic ducts. A similar pattern was seen with 9D5 and in some cases with the pp120 antibody from Transduction Laboratories.

Finally, attempts were made to correlate alterations in expression with other known prognostic markers in breast cancer, including lymph node status, tumor size, steroid receptor expression, and age as well as overall survival. Although there were some general trends sug-

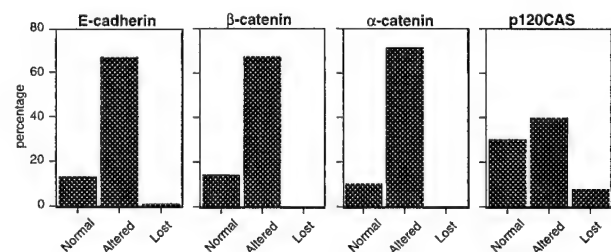


Figure 6. Distribution of expression of each antigen shows a different pattern for p120ctn as compared with the other adhesion molecules.

Table 1. High Correlation of Expression Levels Is Seen between E-Cadherin, α -Catenin, and β -Catenin but Not p120ctn

	E-cadherin	α -Catenin	β -Catenin	p120ctn
E-cadherin		46.3 ($P < 0.0001$)	29.2 ($P < 0.0001$)	0.36 ($P = 0.547$)
α -catenin	46.3 ($P < 0.0001$)		42.2 ($P < 0.0001$)	0.68 ($P = 0.408$)
β -catenin	29.2 ($P < 0.0001$)	42.2 ($P < 0.0001$)		0.04 ($P = 0.835$)
p120ctn	0.36 ($P = 0.547$)	0.68 ($P = 0.408$)	0.04 ($P = 0.835$)	

Each cell shows the χ^2 value and the corresponding P value in parentheses

gesting an association between alteration and poor prognostic factors, no statistically significant associations were found. Similarly, alteration of expression of any of these proteins was not associated with decreased survival. As only p120ctn showed complete loss of expression, we compared this with a combined group of altered and normal expression. We again found no correlation with conventional prognostic markers. As only eight patients fell into this group, it was difficult to meaningfully address survival.

Discussion

Although this is one of the first studies assessing expression of p120ctn in tumors, many studies have examined the other adhesion molecules. Our findings with respect to those studies generally confirm that alteration of expression is a frequent finding. We find 1) alteration of expression is common in invasive carcinoma, but membranous localization is retained, even for β -catenin, in all cases; 2) true loss of expression of E-cadherin, α -catenin, and β -catenin is very rare in ductal carcinoma of the breast; 3) the pattern of expression of E-cadherin, α -catenin, and β -catenin are highly correlated within each case, but do not correlate with expression of p120ctn; 4) there appears to be complete loss of p120ctn in approximately 10% of the cases, although we do not yet know whether this is a function of genetic mutation or down-regulation; and 5) there are general trends correlating alteration of expression of these proteins with traditional poor prognostic markers, but none are statistically significant or independently predictive.

Although use of immunoperoxidase is an accepted standard approach, by using immunofluorescence we attempted to achieve an increased signal-to-noise ratio and more specific subcellular localization. As the role of β -catenin in signal transduction has become better defined,³⁷ localization to the nucleus is suggested and has been described in colonic polyps.³⁶ In invasive ductal carcinoma, nuclear localization is not seen; instead, each case shows membranous localization, although discontinuity and decreased intensity are seen in many cases. In general, we believe alteration is a function of loss of normal differentiation. The discontinuous staining pattern is accounted for by the loss of well defined cell polarity in many tumors. By analogy to observations in the colon, it may be that earlier lesions (ductal carcinoma *in situ* or atypical ductal hyperplasia) need to be examined for nuclear expression of β -catenin, or it may not occur at all in breast cancers.

Our finding that true loss of expression is rare or absent in ductal carcinoma has been observed by others.^{4,9}

Although loss of expression is reported in the literature, it is often grouped with decreased expression,^{5,16} possibly because the methods used are unable to distinguish reduction from true loss.⁶ Molecular analysis to detect mutations in these genes also suggests that loss due to mutation will be rare as mutations in the E-cadherin gene

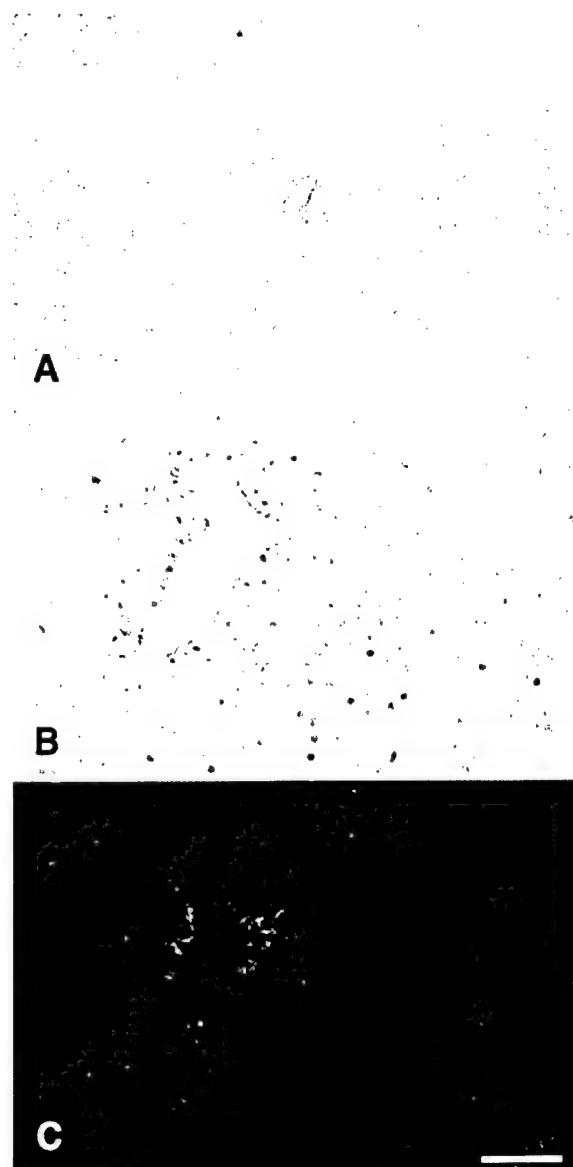


Figure 7. An example of p120ctn staining showing true loss of expression. H&E sections (A and B) show low ($\times 20$) and high ($\times 80$) magnification views of a small duct lined by benign epithelium and surrounded by the cells of an invasive ductal carcinoma. C shows a complete absence of expression of p120ctn in a serial section, with good membranous staining of the entrapped normal duct. Scale bar in C, 50 μ m.

have never been found in ductal carcinoma.⁹ The catenins are less well studied, although preliminary evidence suggests that their loss due to mutation will also be rare in human tumors,¹⁰ even though loss of expression has been described in a number of cell lines.¹⁴

In a previous study using a panel of breast cancer cell lines, there were no examples of loss of expression of p120ctn.³⁰ In contrast, this study shows some clear examples of complete loss of p120ctn in breast cancer tissue sections. One explanation of this finding could be an isoform switch resulting in loss of many epitopes. This seems unlikely because previous studies indicate that MAbs 15D2 and 12F4 recognize all known isoforms of p120ctn.³⁰ An alternative explanation is that the biological processes that are occurring in the tumors with loss of expression represent a stage in the multi-step process of carcinogenesis that is not recapitulated by any cell line yet tested; it is possible that cell lines will eventually be found that show complete loss of p120ctn.

The expression pattern and potential loss of p120ctn has not been previously studied in human breast cancer tissues. Decreased levels of p120ctn expression have been reported in colonic carcinoma³⁸ (preliminary data in our laboratory), bladder cancer,³⁹ and more recently in adenomatous polyps.³⁶ Our study of breast tissue shows reduction or alteration, similar to the colonic cases, but also true loss of expression is seen in a small percentage of the cases. It is unknown whether this loss represents mutation or simply transiently extinguished expression. The fact that some of our cases show areas of loss and other areas with reduction or alteration suggests that the absence of expression may be a result of down-regulation. As the factors that regulate expression of p120ctn are not well understood, we cannot test these cases to distinguish these possibilities. However, consistent with the study in bladder cancers³⁹ and studies on transformed L-cell lines,⁴⁰ it appears that p120ctn is not coordinately regulated with E-cadherin and α - and β -catenin. Future studies will attempt to address the possibility of down-regulation *versus* mutation in these cases.

Acknowledgments

This work is dedicated to patients with breast cancer and their families, including relatives of an author (D. L. Rimm), Ruthy and Robin Brown, Barbara Rimm, and Mary Ruth Reitz.

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Loss of p120ctn in human colorectal cancer predicts metastasis and poor survival

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Received 8 April 1998; received in revised form 16 June 1998; accepted 23 June 1998

Abstract

The p120ctn protein (formerly p120CAS) is an *armadillo* family member that associates directly with the cytoplasmic tail of E-cadherin and participates in the junctional complex responsible for cell–cell adhesion. Since reduced cell–cell adhesion is associated with metastasis in colorectal cancer and other neoplasms, we hypothesize that reduced expression of p120ctn may be related to metastasis in colorectal tumors. Here we describe a study of p120ctn expression in 44 primary human colorectal adenocarcinomas. As detected by immunohistochemical methods, we find altered p120ctn staining patterns in 86% of the cases. Regional complete loss of expression is seen in 18% of the cases, and it correlates with high stage disease and nodal metastasis as well as decreased survival. Although this is a preliminary study, it suggests that downregulation of p120ctn in colon cancer may be associated with metastasis and poor clinical outcome. © 1998 Elsevier Science Ireland Ltd. All rights reserved

Keywords: Catenin; *arm* family; Cadherin; Adhesion; p120CAS

1. Introduction

The 120 kDa cadherin-associated protein, p120ctn, was originally discovered in *c-src* mutational analyses as a protein whose tyrosine phosphorylation correlated with phenotypic transformation [1,2]. P120ctn is tyrosine phosphorylated after stimulation of cells

with growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and colony-stimulating factor 1 (CSF-1), thus implicating p120ctn as a downstream effector of mitogen-induced signaling pathways [3]. Though it was known to be membrane-associated from its discovery, the first evidence linking p120ctn with cell adhesion came when cloning of the gene identified 11 copies of a characteristic 42 amino acid *armadillo* repeat placing it in the *arm* family of proteins [4]. The *arm* family, originally described for the *Drosophila* segment polarity

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gene product armadillo [5], includes the adhesion-related proteins β -catenin and plakoglobin (γ -catenin) [6]. Later co-localization and immunoprecipitation experiments confirmed the idea that p120ctn functions as part of the adhesion complex that contains the cadherins and the catenins [7–9]. P120ctn, like β -catenin and plakoglobin, binds the cytoplasmic tail of E-cadherin directly [10]. However, unlike β -catenin and plakoglobin, p120ctn does not bind directly to α -catenin, nor does it interact with the adenomatous polyposis coli gene product (APC) [10,11].

Adhesion-related molecules are thought to play an important role in carcinogenesis and metastasis. Abnormal epithelial morphology and disorganization of cell–cell adhesion in carcinomas is especially prominent at the borders of a tumor where dedifferentiated cells infiltrate into the surrounding tissue [12,13]. The concept that downregulation of cell adhesion molecules could be responsible for mediating this phenotype was proposed about 50 years ago [14–16]. As metastasis is a complex process known to involve cell detachment and reimplantation at a distant site, there is extensive evidence for involvement by the proteins of cell adhesion [17,18].

Alterations in E-cadherin, α -catenin, β -catenin, plakoglobin and p120ctn have been implicated in the lack of adhesion and increased invasiveness of many types of cell lines and tumors [17,19,20]. In human colorectal cancer, one study showed frequent loss of E-cadherin expression in Dukes stage C compared to Dukes stage A and B disease [21] and a subsequent paper by the same group found lower E-cadherin expression to correlate with worse survival [22]. However, other smaller studies by other groups did not find this association [23,24]. The predictive value of catenin expression has also been evaluated in colorectal carcinoma. Reduction of α -catenin expression correlated better with metastases (to lymph nodes or liver) than reduction of E-cadherin expression [25]. Loss of β -catenin expression has been shown to correlate with high grade tumors [26]. More recently, altered expression of β -catenin (nuclear localization) has been shown in a subset of early lesions [27] and mutations altering adhesion protein interactions have been found in colon cancer cell lines [28]. Furthermore, mutations have been found in β -catenin cancer cell lines [29], but they are uncommon in actual human tumors [30].

While less well characterized than other members of the E-cadherin complex, alterations in p120ctn expression have been shown in cancer. In a study involving human bladder cancers, loss of staining for p120ctn correlated with worse stage, grade and survival, but the correlation between loss of p120ctn and poor survival was less strong than that seen for β -catenin, α -catenin and E-cadherin [31]. In invasive ductal carcinoma of the breast, loss or alteration of p120ctn expression was frequently detected in a pattern unique from that seen for E-cadherin and other catenins suggesting separate regulatory mechanisms [32]. In a study of p120ctn in 13 human colorectal tumors, four had greater than 80% membrane staining of p120ctn (normal), six had between 10 and 80% staining (heterogeneous) and three had less than 10% expression (complete loss) [33]. There was a general trend between p120ctn expression and E-cadherin expression, although no significant correlation was found. There was a correlation between loss of p120ctn and larger tumor size when the group of normal expressors was compared to the combined heterogeneous and complete loss groups. No correlation could be found with either distant metastasis or metastasis to the lymph nodes in this small group.

Here we describe a study of p120ctn expression in human colorectal carcinoma and potential clinicopathological correlations. Given the relation of the loss of p120ctn expression with high grade, high stage and decreased survival in bladder cancer, we hypothesize that alteration in p120ctn expression may correlate with poor outcome in colorectal cancer.

2. Materials and methods

2.1. Patients and tumor specimens

Forty-four formalin-fixed paraffin-embedded tissue blocks were obtained from patients who underwent curative resections for colorectal cancer in 1993 and 1994 at Yale-New Haven Hospital. Blocks were selected by viewing original pathological slides and choosing blocks that showed the junction between normal colonic epithelium and tumor. Clinical information corresponding to the tissue blocks was obtained from the Yale-New Haven Hospital pathology database and the Yale-New Haven Hospital

Tumor Registry. Patient follow-up data including recurrence data and survival times for each patient were obtained with assistance from Dr Diana Fisher at the Yale-New Haven Tumor Registry. The patient population ranged in age from 40 to 90 years with a median age of 67 years. The median follow-up time on this population was 24 months (mean 29 months). All information and material was collected and used in accordance with the Yale Human Investigation Committee Protocol #8219 (D.L.R.).

2.2. Antibody

The p120ctn-specific monoclonal antibody 12F4 was chosen from a panel of p120ctn-specific monoclonal antibodies (Wu and Reynolds, unpublished data) because of its excellent reactivity and low background with formaldehyde-fixed tissue sections [32]. This antibody binds to an epitope in the carboxy-terminal 121 amino acids of p120ctn and recognizes all known isoforms of p120ctn that can be distinguished by immunoprecipitation and Western blotting analysis. The reagent used was affinity purified from ascites on protein A sepharose columns.

2.3. Immunohistochemical analysis

Immunohistochemical analysis of the standard histologic sections was done as described previously [32]. Briefly, sections were cut from the paraffin blocks, baked overnight at 60°C and deparaffinized. For antigen retrieval, each slide was immersed for 5 min in boiling 6.5 mM sodium citrate (pH 6.0) in a conventional pressure cooker [34]. After a 1 h incubation in 0.3% BSA (Sigma, St. Louis, MO) diluted in TBS (pH 8.0) to block non-specific binding, the slides were incubated for 1 h with the monoclonal antibody 12F4 diluted 1:500. The slides were then washed seven times and incubated for 1 h with Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:500 in TBS (pH 8.0). They were then washed and coverslipped with *N*-propyl galate, sealed with nail polish and stored at -20°C.

2.4. Histological scoring and analysis

All slides were independently examined by two

individuals (J.S.G. and D.L.R.) on an Olympus AX-70 epifluorescence photo-microscope. For photography of corresponding sections, immunosections were marked with a cytologists pen and then aligned to the H&E section which was marked for photography. The internal control regions of normal colonic epithelium were assessed for strong uniform staining. The tumors were compared to the control regions. Observation of a number of cases showed that p120ctn expression was lost in some regions of the tumors, but not others. Therefore, a score was assigned for the expression of p120ctn both in the entire tumor seen on each slide and in a 40 × field that was chosen to represent the worst staining region of each slide (i.e. the area in which there is the greatest loss of p120ctn expression). Epithelium with greater than 75% staining was scored as 'normal', that with between 75 and 10% was scored as 'altered' and that with less than 10% was scored as 'loss'. There was 87.5% agreement between the two observers and in no case was there a discrepancy in scoring such that one observer scored as 'normal' what the other deemed 'loss'. Consensus scores were then assigned for each case by reviewing the slides with discrepancies in scoring. In order to make the score 'loss' stringent, it was not assigned as the consensus score unless both observers independently assigned this score. Examples of areas for each scoring category are shown in Fig. 1.

Data analysis was accomplished using StatView 4.5 for Macintosh (Abacus Concepts, Berkeley, CA).

3. Results

3.1. Many tumors show alteration or loss of p120ctn expression

Standard pathological slides cut from paraffin-embedded blocks selected to contain both colorectal cancer and benign epithelium were stained using a monoclonal antibody directed against p120ctn followed by a Cy3-conjugated secondary antibody. In the vast majority of slides, the tumor could be compared with an adjacent area of normal colonic epithelium that served as an internal control for p120ctn staining. The normal colonic epithelium uniformly stained strongly for p120ctn in a typical membranous pattern as described previously [33]. Unlike normal

epithelium, the expression of p120ctn in colorectal tumors was variable. A scoring system was devised to account for this variation. The expression of p120ctn in each tumor was quantified by the approximate percentage of the cell membranes in which

staining was seen. Since there was often variation in expression of p120ctn between different regions of a given tumor, the scoring system, as described in Section 2, was done on the $40\times$ field with the lowest level of expression on the section examined. The

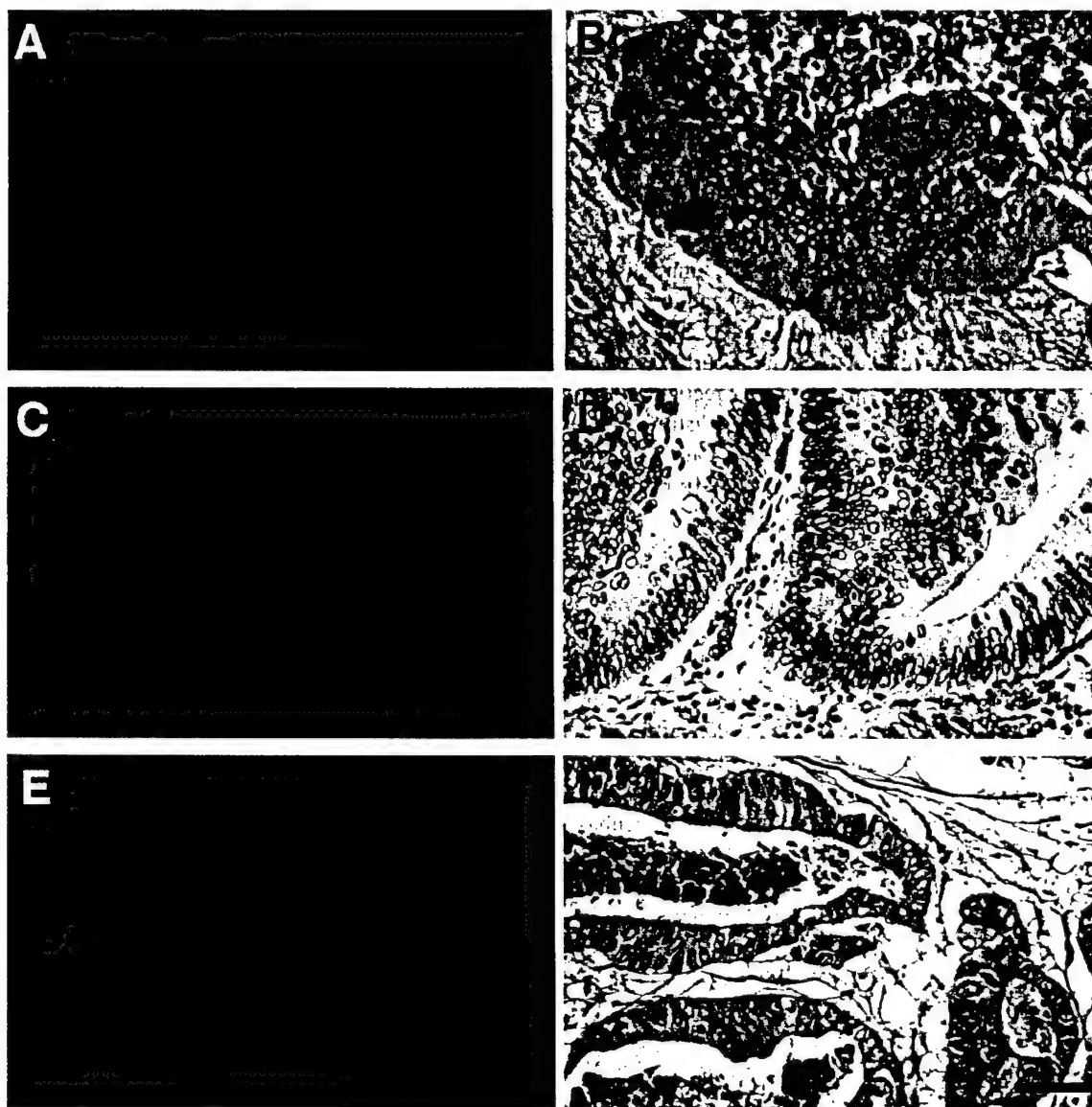


Fig. 1. The scoring system for assessment of p120ctn expression is illustrated by representative cases. (A) An example of staining indistinguishable from normal non-malignant tissue. (C) An example of a region scored as altered staining but not complete loss. (E) An example of a region of complete loss. The morphology of the corresponding regions from serial sections three to five sections (15–25 μm) deeper are shown by H&E staining of serial sections in (B,D,F). Thus, the same region of the section is represented, although the architecture is somewhat different. Note the same section cannot be shown since eosin is a fluorescent dye. The scale bar in (F) is equal to 50 μm .

Table 1

Summary of histopathologic data and p120ctn staining of cases analyzed

Number	Tumor location	Tumor size (cm)	Grade	Stage	Node status	Presence of lymphovascular invasion	Presence of mucinous or signet ring features	Score
1	Right colon	5.0	2	I	Negative	No	No	Altered
2	Right colon	2.2	2	II	Negative	No	No	Normal
3	Right colon	4.5	3	III	Positive	Yes	Yes	Loss
4	Right colon	4.5	2	I	Negative	No	No	Altered
5	Right colon	4.0	2	II	Negative	No	No	Altered
6	Right colon	4.0	2	IV	Positive	No	No	Altered
7	Right colon	4.5	2	II	Negative	No	No	Altered
8	Right colon	5.0	2	II	Negative	No	No	Altered
9	Right colon	6.0	2	III	Positive	No	No	Loss
10	Right colon	2.5	2	III	Positive	No	No	Altered
11	Right colon	10.0	3	II	Negative	No	Yes	Altered
12	Right colon	9.0	2	IV	Positive	Yes	No	Loss
13	Right colon	5.0	3	IV	Positive	Yes	No	Loss
14	Right colon	3.5	2	II	Negative	No	No	Altered
15	Right colon	3.2	2	I	Negative	No	No	Loss
16	Rectum	4.0	2	IV	Positive	Yes	No	Loss
17	Sigmoid	6.0	3	IV	Positive	Yes	No	Normal
18	Right colon	3.8	2	III	Positive	Yes	No	Normal
19	Right colon	3.0	2	II	Negative	No	No	Altered
20	Left colon	7.5	2	II	Negative	Yes	No	Altered
21	Right colon	2.0	3	IV	Positive	Yes	No	Altered
22	Rectum	3.5	2	III	Positive	Yes	No	Altered
23	Unknown	3.5	2	II	Negative	No	No	Altered
24	Left colon	5.0	2	III	Positive	Yes	No	Altered
25	Rectum	2.0	2	I	Negative	No	No	Altered
26	Right colon	4.0	2	III	Positive	No	No	Loss
27	Right colon	2.5	2	IV	Positive	No	No	Altered
28	Unknown	13.0	3	IV	Positive	Yes	Yes	Altered
29	Right colon	3.5	2	II	Negative	Yes	No	Altered
30	Sigmoid	5.0	2	IV	Negative	Yes	No	Altered
31	Right colon	7.0	3	III	Positive	Yes	Yes	Altered
32	Rectum	4.0	2	II	Negative	No	No	Normal
33	Unknown	?	3	III	Positive	No	No	Altered
34	Right colon	2.0	2	I	Negative	No	No	Altered
35	Sigmoid	3.0	2	II	Negative	Yes	No	Altered
36	Right colon	4.5	2	II	Negative	No	No	Normal
37	Unknown	2.0	2	III	Positive	No	No	Normal
38	Sigmoid	4.0	2	III	Positive	Yes	No	Altered
39	Right colon	8.0	3	II	Negative	No	No	Altered
40	Left colon	5.0	2	II	Negative	No	No	Altered
41	Right colon	6.0	2	II	Negative	Yes	No	Altered
42 ^a	Left colon	4.0	2	III	Positive	No	No	Altered
43 ^b	Left colon	3.0	2	II	Negative	No	No	Altered
44 ^c	Left colon	5.0	3	III	Positive	Yes	Yes	Loss

^aThis patient had a bowel resection for colorectal cancer about 20 years before this tumor was diagnosed.^bThis tumor is a recurrence of a colorectal cancer that was resected 439 days before this tumor was resected.^cTwo slides from this case were scored separately and the score shown represents the worse of the two.

region with the lowest level of staining was used for scoring purposes because it was hypothesized that if loss of p120ctn promoted invasion, loss of this protein in one region of a tumor could provide a clonal focus with metastatic potential, even if the rest of the tumor was relatively spared.

The tumors were thus divided into three groups, i.e. those with normal, those with altered and those with loss of p120ctn expression in the region with the most abnormal staining. Examples for each score are shown in Fig. 1. It should be noted that the percentage of the total cell membranes in which there was appreciable staining, regardless of intensity, was used for scoring purposes. Therefore, the intensity of the staining is not reflected in the scoring system despite the fact that the tumors on the whole had less intense staining than the adjacent normal tissue, even when the expression was scored as normal. The scores from 44 cases and the pathological data from each patient are shown in Table 1.

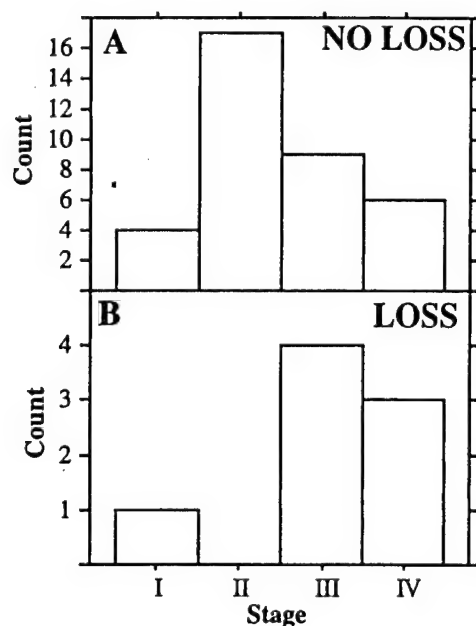


Fig. 2. Distribution of cases by stage shows that most of the cases with normal or altered p120ctn expression are AJCC stage I and II, whereas most of the cases with regional loss of staining are stage III and IV. Histograms show division into groups with normal or altered p120ctn expression (A) or regional loss of p120ctn (B). Slides were scored for p120ctn expression as described in Section 2.

Table 2

Contingency table of stage versus loss of p120ctn expression

	Normal/altered Loss		Total
Low (I or II)	21	1	22
High (III or IV)	15	7	22
Total	36	8	44

$\chi^2 = 5.500$; Fischer's exact *P*-value 0.0459.

3.2. Loss of p120ctn expression correlates with metastasis

To test the hypothesis that areas of loss may be associated with metastasis, the altered and normal cases were combined and compared with the cases with regional loss of expression. Two significant correlations with metastasis were seen. First, loss of p120ctn staining is significantly associated with AJCC stage III or IV disease, i.e. cancer that had metastasized to either the lymph nodes or a distant organ ($P = 0.0459$). The distribution of cases by stages for tumors with or without an area of loss of p120ctn expression can be seen in Fig. 2. Regional loss of p120ctn is more commonly seen in advanced stage disease than in low stage tumors. A contingency table constructed from these data is shown in Table 2.

Similarly, cases with regional loss of p120ctn expression also correlate with lymph node metastasis ($P = 0.0190$). A contingency table showing the high proportion of node-positive cancers with loss of p120ctn expression and the low proportion of node-negative cancers with such loss can be seen in Table 3. The vast majority of tumors with an area of loss of p120ctn expression were metastatic by either of these two measures (seven of eight cases). Of the 23 cases with metastases (21 to lymph nodes at diagnosis and two node-negatives with later distant metastases),

Table 3

Contingency table of nodal status versus loss of p120ctn expression

	Normal/altered Loss		Total
Negative	22	1	23
Positive	14	7	21
Total	36	8	44

$\chi^2 = 6.200$; Fischer's exact *P*-value 0.0190.

seven showed loss, 13 were altered and only three were normal.

3.3. Loss of p120ctn expression correlates with both shorter overall survival and recurrence-free survival

We found that regional loss of p120ctn expression correlates with decreased survival. Although the cohort is small, statistical analysis showed significance (Mantel–Cox log-rank P -value 0.0079). A Kaplan–Meier life table analysis of this association is shown in Fig. 3. Four patients had a tumor recurrence, and in all cases the recurrence was metastatic as opposed to local. As with overall survival, shorter recurrence-free survival also correlated with regional loss of p120ctn ($P = 0.0214$). Both survival and disease-free survival are calculated from the date of the operation from which the tissue analyzed was obtained. The Kaplan–Meier life table analysis is shown in Fig. 4.

Other possible associations were examined but could not be found. Specifically, the expression of p120ctn did not correlate with either patient age or gender or with the location of the tumor within the large intestine (right colon, left colon, sigmoid or rectum). Associations were similarly not found between p120ctn expression and tumor histological grade,

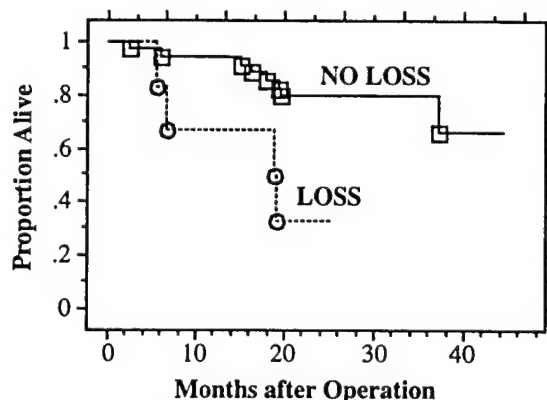


Fig. 3. Kaplan–Meier life table analysis shows that overall survival was significantly worse for the patients whose tumors had regional loss of p120ctn compared to those with normal or altered expression (log-rank $P = 0.0079$). The profile for the group with no regional loss of expression, labeled NO LOSS, is shown by a solid line with each event indicated by a square. Eight of 36 patients in this group died. Those with regional loss, labeled LOSS, are indicated by the broken line with a circle for each event. Four of eight patients in this group died.

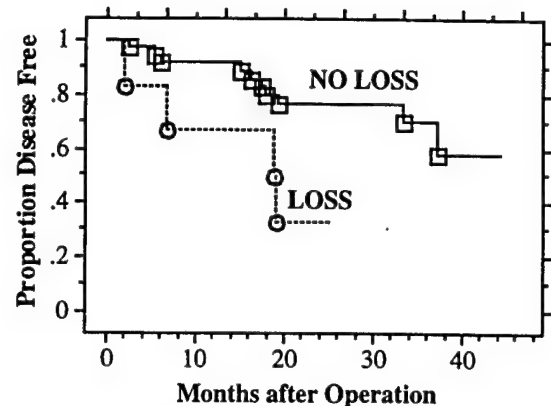


Fig. 4. Kaplan–Meier life table analysis shows that disease-free survival was significantly worse for the patients whose tumors had regional loss of p120ctn compared to those with normal or altered staining (log-rank $P = 0.0214$). The profile for the group with no regional loss of expression, labeled NO LOSS, is shown by a solid line with each event indicated by a square. Ten of 36 patients in this group recurred or died. Those with regional loss, labeled LOSS, are indicated by the broken line with a circle for each event. Four of eight patients in this group recurred and died.

mucinous or signet ring features, or lymphovascular invasion as documented on the pathology report. Multivariate analysis was attempted, but due to our small sample size we were not able to assess the independent predictive value of regional loss of p120ctn expression.

4. Discussion

The data presented here confirm and extend the results of a previous study showing that alteration of p120ctn expression is a frequent event in colorectal carcinoma [33]. The larger number of patients in this series and a scoring system based on regional loss of p120ctn, however, suggests additional correlations to outcome that were not previously reported. Specifically, cancers with regional loss of p120ctn expression are more likely to be metastatic and result in poor survival.

As the exact function of p120ctn within the E-cadherin complex is not understood, the significance of these findings cannot yet be fully appreciated. For instance, it is not known whether loss of p120ctn from the cadherin complex would be expected to directly cause loss of adhesion and metastasis. It may be that the loss of p120ctn seen in this study

represents an underlying dysfunction of the cadherin complex and is an outcome rather than a cause of the loss of adhesion. Since E-cadherin expression was not analyzed in this study, it cannot be said with certainty that loss of p120ctn is not simply a marker for loss of E-cadherin at the membrane and hence loss of a site for p120ctn to bind. Skoudy et al. [33] note E-cadherin-negative colorectal tumors where p120ctn localization is diffuse. On the other hand, a study of bladder cancer found normal p120ctn expression at the membrane in some cases where E-cadherin expression was absent [31]. In breast cancer, expression of p120ctn and E-cadherin has been shown to be independently expressed [32]. Finally, these data are complicated by the fact that p120ctn binds to other cadherins such as P-cadherin and N-cadherin, which might target p120ctn to the membrane, even in the absence of E-cadherin.

In this study, the majority of metastatic tumors were not observed to have an area of complete loss of p120ctn. One complication of these data is that the sections analyzed represent only a small fraction of the entire tumor. For example, in one case with metastatic disease, two slides were examined, but only one slide revealed a 40 \times field that could be scored as having regional loss. Many tumors in this study were large masses and it would be impossible to completely sample each tumor. Thus, only one section was examined in each case, regardless of the size of the colorectal tumor. It is possible that if these cases with metastasis were more extensively sampled, regions of loss might be found. Although further studies are planned to systematically sample entire tumors, we tentatively conclude that focal complete loss of p120ctn is associated with metastasis.

While focal loss of p120ctn correlated with metastasis and poor survival in colorectal carcinoma, overall p120ctn expression as scored in this study did not. Our results were based on a scoring system designed to assess regional loss of p120ctn. A set of scores that were simultaneously generated based on the expression of p120ctn throughout the tumor (data not shown) did not yield any significant correlations. This finding is consistent with the hypothesis that focal disruption of cell–cell adhesion is necessary for metastasis.

In summary, this study shows that alteration of p120ctn expression is a common event in colorectal

carcinoma. We show that regional loss of p120ctn is related to metastasis in colorectal cancers and that loss of p120ctn predicts poor survival. The relatively small sample size and limited follow-up time precluded the assessment of the independent predictive value of loss of p120ctn expression. Further studies will be needed to determine if loss of p120ctn is a cause of increased invasiveness or if it simply represents poor function of the cell–cell adhesion complex in which it functions. Additional studies will also be needed to determine whether loss of p120ctn is truly an independent predictor of poor survival.

Acknowledgements

This work was supported by grants from the William and Catherine Weldon Donaghue Foundation for Medical Research and the US Army (DAMD17-94-J4366).

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Polymerase Chain Reaction-Based Detection of Clonality as a Non-Morphologic Diagnostic Tool for Fine-Needle Aspiration of the Breast

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Presented at the American Society for Cytopathology Meeting, Boston, Massachusetts, November 1-4, 1997.

Supported by grants from the William and Catherine Weldon Donaghue Foundation for Medical Research and the U. S. Army (DAMD17-94-J4366) to David L. Rimm.

The authors thank Tom D'Aquila for assistance with cataloging and organization of the Yale Critical Technologies Cytology collections.

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Received December 22, 1997; revision received March 27, 1998; accepted April 2, 1998.

BACKGROUND. Fine-needle aspiration (FNA) of breast specimens can be difficult and between 10-25% of the lesions ultimately are classified as "atypical," even by the most experienced cytopathologist. The goal of this study was to identify a molecular mechanism that reliably distinguishes benign and malignant (or pre-malignant) lesions and that could be used as an adjunct in these morphologically ambiguous cases.

METHODS. Because all malignancies represent clonal proliferations, assessment of clonality represents a potential molecular mechanism for making this distinction. Excess material preserved from breast FNAs was examined using the human androgen receptor locus clonality assay. This assay allows determination of clonality on the basis of X chromosome inactivation as detected by polymerase chain reaction analysis of genomic DNA after methylase-sensitive restriction digestion.

RESULTS. In this pilot study, 25 cases showed reproducible results. All malignant cases (9 of 9) were monoclonal, whereas 10 of 12 benign cases were polyclonal. Of four atypical cases, two were monoclonal and both were found to be malignant after surgical resection. Monoclonality was observed in two benign cases that were hyperplastic lesions.

CONCLUSIONS. These preliminary results suggest that this test may provide a non-morphologic molecular mechanism for the objective categorization of breast FNAs. *Cancer (Cancer Cytopathol)* 1998;84:262-7.

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KEYWORDS: fine-needle aspiration, carcinoma, human androgen receptor (HUMARA), hyperplasia, polymerase chain reaction.

From a molecular perspective, fine-needle aspiration (FNA) represents a relatively untapped and potentially highly valuable modality. One of its unique properties is that the mechanophysical procedure used to obtain the material results in the preferential harvest of epithelial cells over the stromal background. Diagnosis of breast malignancy should be achievable by examination of these epithelial cells and in general a trained cytopathologist can make a definitive morphologic diagnosis in 70-90% of the cases.¹ However, it would be desirable to develop a molecular assay that could detect biologic alterations that would allow definitive diagnosis of malignancy, even without morphologic inspection. This would not only be useful to resolve morphologically difficult cases, but it could allow the process to become more objective and perhaps ultimately even automated.

Molecular diagnosis in breast carcinoma has been difficult because there is no universal marker. A number of markers have been examined, but none are mutated or specific for even 70% of breast

carcinomas. Although it is not a mutation, one biologic phenomenon present in 100% of breast carcinomas is that, by definition, they are clonal. It has been shown that polymerase chain reaction (PCR) of microdissected breast carcinoma specimens can be used to detect clonality² and that common benign lesions (fibroadenoma) are polyclonal in contrast to the morphologically similar phyllodes tumor.³ In another study, samples from multiple sites in the same breast showed inactivation of the same allele, suggesting that the neoplasm had a clonal origin, and what appeared multicentric most likely was tumor spread through the path of least resistance (along the ducts).⁴ Furthermore, clonality even has been suggested as a potentially useful modality to distinguish early premalignancy from benign hyperplasia.^{5,6}

Lyon⁷ showed that the random inactivation of either the paternal or the maternal X chromosome occurs during early embryogenesis of females and the progeny of these cells retain this same inactivated X chromosome. When polymorphisms occur on the X chromosome the cellular population becomes a mosaic for the activated alleles. Allen et al.⁸ defined a method with which to detect X inactivation and thus determine the monoclonality of a given sample on the basis of the inactivated X chromosome. The human androgen receptor (HUMARA) locus clonality assay entails isolation of DNA and PCR amplification around a highly polymorphic trinucleotide repeat in the first exon. Two product bands may be visualized, representing the maternal and paternal alleles. In a parallel PCR reaction, the template is predigested with HpaII (or HhaI), which cuts the DNA between the sites to be amplified by the PCR primers. Because the inactive X chromosome is methylated, it is not cut, and PCR occurs normally. On the active X chromosome, the DNA is cut and no PCR product results. These PCR pairs then are resolved by electrophoresis and the "uncut" lane shows two bands if the case is informative. The "cut" lane also will show 2 bands if the lesion is polyclonal, because there is approximately 50% inactivation of each chromosome. However, if the lesion is clonal, then only a single band will be observed, migrating with one of the two bands in the adjacent lane. This technique previously has been well summarized and illustrated in more detail elsewhere.⁹

In this article, we described the application of the HUMARA assay to excess material collected from breast FNAs. To date we have completed analysis of 37 specimens. We report the outcome of 25 informative cases, including comparisons with cytologic diagnoses and follow-up surgical diagnoses, when available.

MATERIALS AND METHODS

Tissue Acquisition

Tumor samples were obtained through the Cytology Division of the Critical Technology Program at the Yale University School of Medicine in accordance with Yale Human Investigation Committee Protocol 8219. The material used in these studies is taken after diagnosis, and otherwise would be discarded. It was stored in methanol-based PreservCytTM solution (Cytoc Corporation, Boxboro, MA) for 1 month to nearly 2 years before preparation of DNA. The Thin PrepTM (Cytoc Corporation) slide made for diagnostic purposes was used for morphologic examination of the material. All samples were breast FNA biopsy tissue removed between June 1995 and July 1996. The median age of the patients was 50.8 years (range, 22–90 years). Cases with excess material were selected serially by presentation date, starting in June 1995. Cases that had insufficient material in the needle washings for production of DNA were omitted. Of 37 cases selected, 35 had sufficient DNA for a reproducible evaluation. Of these, 25 gave an informative result with respect to clonality. The distribution of cases by diagnosis is shown in Table 1.

DNA Extraction

Material was collected by drawing cells from the vial. Cells in 1–20 mLs were sedimented to remove the PreservCytTM and resuspended in an appropriate amount (30–300 mL) of digestion buffer containing 10 mM Tris (pH 8), 100 mM NaCl, 25 mM ethylenediamine tetraacetic acid, 0.5% sodium dodecyl sulfate, and proteinase K to 100 mg/mL. Each specimen was incubated overnight at 37 °C before extraction in an equal volume of phenol/chloroform. DNA was extracted once, precipitated with cold ethanol, washed once, and resuspend in varying amounts of TE buffer (15–50 mL).

DNA Quantitation

Genomic DNA of known concentration was diluted to construct a standard curve by the addition of series of concentrations in 50 mL of TE to 50 mL of TE containing 10× SYBR Green I (FMC BioProducts, Rockland, ME). Specimen DNAs were diluted similarly. Samples were set up on a 96-well plate and fluorescence was quantitated on a Fluostar plate reader (TECAN Inc., Research Triangle Park, NC) using a 485-nanometer (nm) excitation filter and a 520-nm emission filter. Fluorescence versus concentration of known samples was plotted to produce a standard curve and specimen DNA concentrations were determined.

TABLE 1
Summary of Informative Cases

Case no.	Clonality	No. of reps	Cytol Dx	Surg Dx
1	M	2	B	ND
3	P	3	B	ND
5	M	2	B	B
6	M	3	Malig	Malig
8	M	2	Malig	ND
11	P	2	B	ND
12	M	2	A	Malig
17	P	2	B	B
21	M	2	Malig	Malig
22	P	3	B	B
24	P	2	A	B
26	P	2	B	ND
30	M	2	Malig	Malig
31	M	2	Malig	Malig
34	P	2	A	Malig
36	P	3	B	ND
39	P	2	B	ND
40	M	2	Malig	Malig
41	M	2	Malig	Malig
42	M	3	Malig	Malig
43	P	2	B	ND
45	M	2	A	Malig
48	P	2	B	ND
58	M	2	Malig	Malig
66	P	2	B	ND

No. of reps: number of times the assay was repeated to prove clonality status; Cytol Dx: cytologic diagnosis; Surg Dx: surgical/histologic diagnosis; M: monoclonal; P: polyclonal; B: benign; ND: no surgery done; Malig: malignant; A: atypical.

Polymerase Chain Reaction

Between 30–60 ng DNA was incubated at 37 °C for 1 hour in the presence or absence of HhaI or HpaII (10 U/reaction; New England Biolabs, Beverly, MA), the accompanying buffer, and water to 10 mL. Samples then were heated to 65 °C for 20 minutes to inactivate the enzymes. This material was added to a PCR reaction (final volume: 50 mL) for amplification with the primer pair. Primers used were: 5'GCTGTGAAGGTT-GCTGTTCTCAT3' and 5'TCCAGAATCTGTTCCA-GAGCGTGC3'.

Each reaction contained 20 pmol of each primer, 10 nmol of each deoxynucleotide triphosphate, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM MgCl₂, and 1.5 U of Amplitaq Gold[™] Taq polymerase (Perkin-Elmer, Oak Brook, IL). Thermal cycling was performed in a PTC-100[™] Programmable Thermal Cycler (MJ Research Inc., Watertown, MA). An initial denaturing step of 10 minutes at 95 °C was used to activate the Taq polymerase. Three cycles followed starting with 95 °C for 4 minutes, 58 °C for 45 seconds, and 72 °C for 1 minute and 30 seconds. Twenty-eight additional cycles were used with 95 °C for 30 seconds, 58 °C for 45

seconds, and 72 °C for 1 minute and 30 seconds, ending with a 7-minute extension step at 72 °C.

Detection and Scoring of Clonality

PCR products were run out on 6% nondenaturing polyacrylamide gels, stained with SYBR Green I, and viewed under ultraviolet (UV) light. Some samples were rerun on an 8% denaturing polyacrylamide gel to obtain better band separation. These were stained with SYBR Green II and viewed under UV light. Samples that were restriction digested then were compared with undigested samples. A small subset of the cases were amplified with ³²PdCTP in the deoxynucleotide mix to enhance sensitivity. Radioactively labeled samples were resolved on denaturing gels and dried before exposure. Using radioactivity allowed us to decrease the template amount from 30–60 ng to 10–20 ng. This was successful in resolving bands that were not resolved using the SYBR Green I method in a few cases, but this method was not exhaustively optimized in this study.

Cases were scored as "informative" if two bands were visible in the undigested lane. If both bands were present at similar intensities to the undigested lane, cases were scored as "polyclonal." Cases were scored as "monoclonal" if one of the two bands disappeared after restriction enzyme digestion. The criteria for inclusion in the study was at least two separate assays showing the same result. The criteria for monoclonality was qualitative significant decrease in the intensity of either the upper or lower allele compared with the intensity of the undigested control lane.

RESULTS

Determination of clonality was attempted on all samples from which sufficient DNA could be produced, including obvious malignancies, obvious cases of benign or reactive conditions, and atypical cases. Figure 1 illustrates the assay for determination of clonality. Lanes labeled (U) should show two bands in each case. If they are sufficiently resolved, then the case is informative. The other lane in each pair (C) is that cut with a restriction enzyme before PCR and should show one band if the sample is monoclonal or two bands if it is polyclonal. Case 8 is an example of a clonal neoplasm, whereas Case 17 is an example of a polyclonal benign specimen. Panel B shows some additional examples of cases that were more difficult to interpret. Case 3 was found to be polyclonal, although this example showed an assay in which insufficient template was used and the clonality could not be determined. Case 41 showed an example of a monoclonal case, with loss of the larger allele (rather than the smaller one as observed in Case 8). Finally, Case 38 shows an

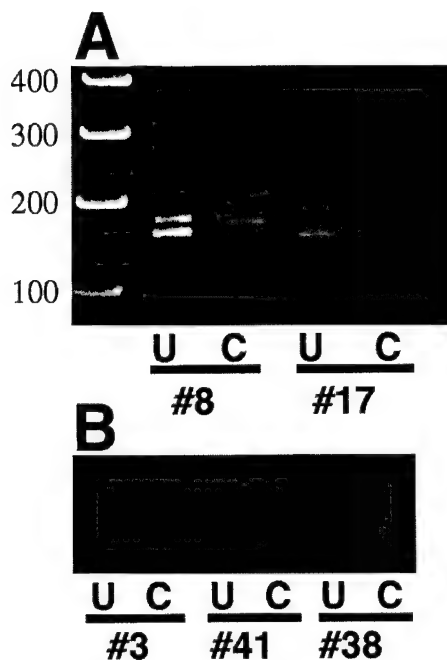


FIGURE 1. Examples of the method of determination of clonality. (A) Two cases are shown next to a size marker resolved by nondenaturing gel electrophoresis in 6% acrylamide gels. The size markers are labeled in the far left lane. The expected product size was approximately 180 base pairs. Each case number is shown below two lanes. U: uncut by restriction enzymes; C: cut by restriction enzymes. Case 8 is an example of a clonal neoplasm, whereas Case 17 is an example of a polyclonal benign specimen. Panel B shows some additional examples of cases that were more difficult to interpret. Case numbers are shown beneath each set of lanes.

example of an uninformative case in which the difference in the two alleles was insufficient for resolution by our system of analysis.

Table 1 shows the distribution of cases showing the outcome of the clonality assay including the number of confirmatory repetitions and the cytologic and surgical diagnoses. These were determined by analysis of clonality using the HUMARA assay described earlier with either nonradioactive (Fig. 1) or radioactive DNA visualization of the PCR products. To date, analysis has been completed on 37 cases, 25 of which were reproducible and informative. The clonality results are summarized by cytologic diagnosis in Table 2.

The majority of the cases in this pilot study were either clearly benign or malignant, but four cases were considered morphologically atypical, resulting in surgical removal of the lesion. Representative morphologic images from each of these cases are shown in Figure 2. In three of the cases, the clonality correctly predicted the surgical diagnosis (Figs. 2A-C). Two of three resected cases that were found to be malignant (Cases 12 and 45) were monoclonal and one atypical

TABLE 2
Summary of Clonality by Cytologic Diagnosis

Cytol Dx	Polyclonal	Monoclonal	Total
Benign	10	2	12
Atypical	2	2	4
Malignant	0	9	9
Total	12	13	25

Cytol Dx: cytologic diagnosis.

case was surgically proven to be benign (Case 24); this case was polyclonal. One case (Case 34) was polyclonal but found to be malignant by surgical biopsy. It was termed "atypical" because it showed high cellularity and single atypical-appearing cells mixed with many benign cells (Figs. 2D-F). In this type of case, the clonality assay appears to be ineffective.

DISCUSSION

All cases that had a cytologic diagnosis of malignancy were monoclonal by the HUMARA clonality assay. Only one case with a surgical diagnosis of "malignant" and a cytologic diagnosis of "atypical" was found to be polyclonal, and this case (as illustrated earlier) was not a good candidate for this assay due to an abundant benign ductal cell and inflammatory cell component. Benign cases were less definitive. Two cases that were benign cytologically were shown to be monoclonal. There was surgical follow-up on one case, showing ductal hyperplasia. The second case did not have surgical follow-up, although hyperplasia was suggested morphologically in the cytologic specimen.

This outcome raises the question of the minimal clonal unit in breast tissue and the earliest lesion that represents a clonal proliferation. The minimal clonal unit perhaps is best described for gut tissue. Using transgenic mice, Hermiston et al. have shown that one crypt and one quarter of each adjacent villus represents a single clonal unit¹⁰ Others have shown similar results by the HUMARA assay.¹¹ The breast represents a less easily defined structure and thus it has been more difficult to make the analogous determination. There is some evidence that the clonal unit is relatively small (possibly a single lobule) and there is clear representation of both X alleles within each breast.¹² Our work is consistent with these data because many benign cases in the current study were found to be polyclonal. Regardless of the size of the minimal clonal unit in normal breast, it appears to be small enough for a standard FNA procedure to obtain material of polyclonal origin. This may be due not only to the size of the unit but also to the relative amount and

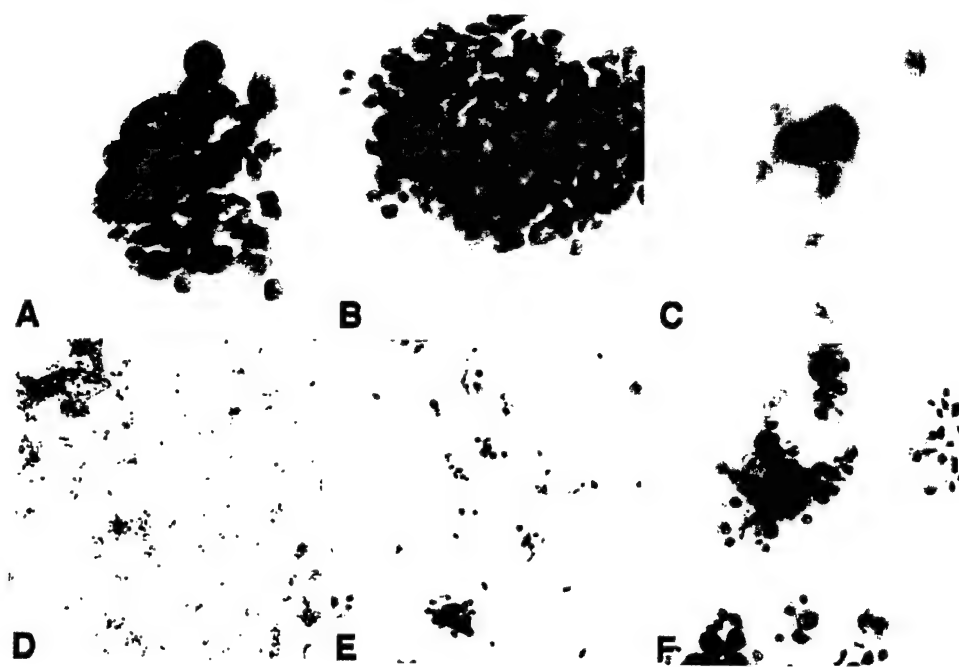


FIGURE 2. Micrographs of the atypical aspirates. Four of the 25 cases analyzed were considered "atypical" by the cytopathologist responsible for the case. (A and B) Two cases were monoclonal and found to be malignant by surgical biopsy ($\times 60$ objective lens). (C) The third case was found to be benign by surgical biopsy ($\times 60$ objective lens). (D-F) One case was polyclonal but was found to be malignant by surgical biopsy ($\times 10$, $\times 20$, and $\times 40$ objective lenses, respectively). It appears that the specimen included a number of benign as well as malignant cells.

origin of the tissue obtained. For example, in benign fibrous lesions, it generally is true that the stromal cell to epithelial cell ratio is much higher, even though the cellularity generally is lower.

The question of what represents the earliest premalignant lesion may represent a more difficult problem. A clonal expansion does not necessarily define malignancy or even premalignancy, although all malignant lesions must begin as clonal expansions. Atypical ductal hyperplasia is considered by most pathologists to be a premalignant lesion. It also has been shown to be monoclonal.¹³ However, even "benign proliferative disease" or benign ductal hyperplasia appears to represent an expansion of a single clone that is sufficiently large to be detected by this assay (at least in two cases in the current study). Although there is little evidence that these are "pre-malignant conditions," they do carry a small but significantly increased risk for significant disease in the future.¹⁴ Depending on the goal of the assay, true "pre-malignancy" may not be important. Many times, the degree of uncertainty in proliferative conditions results in surgical biopsy anyway. Arguably, clonality by this assay, even in the absence of morphologic atypia, may suggest biopsy.

Use of FNA material in this assay raises the question of the amount of tumor required and the tolerance for nonmalignant tissue also present in the spec-

imen. This is a difficult question to address because FNAs vary in the components represented depending on a range of variables related to the lesion, the operator, and other factors. Overall, it is believed that FNAs are enriched in tumor tissue, possibly due to the less cohesive nature of malignant cells. In attempts to address this issue, we performed mixing studies with DNA from male nonbreast specimens (which show only one band). We found that differing sized alleles could be detected easily at the 10% level (data not shown). Because we use the undigested control as a standard in these breast cases, it is possible that we could detect monoclonality if as little as 10% of the cells represented tumor cells. Conversely, we found that one case that was termed atypical on cytology was found to be malignant at surgery and was polyclonal by this assay. In this case 10–20% of the cells appeared morphologically malignant. The true number or minimal percentage of malignant cells required for a successful assay remains to be determined precisely.

The clonality assay, as described earlier, was less robust than expected. We were unable to interpret 12 of 37 cases. Of these, two cases were uninterpretable due to insufficient amounts of DNA for duplicate harvesting and analysis and ten were uninterpretable due to insufficient resolution of the alleles. The system we used to resolve the differences in alleles most likely was only sufficiently sensitive when alleles varied by

more than three repeats. Although we found a total of 27 of 37 cases to be informative (73%); other groups have estimated that this assay should be informative in approximately 90% of cases. Other more sensitive systems are described elsewhere¹⁵ and currently are under development in our laboratory.

The use of clonality as an ancillary technique in diagnostic pathology was pioneered in hematopathology. It was first performed using assessing immunoglobulin heavy chain rearrangement by Southern blot analysis and later by assessing both immunoglobulin heavy chain and T-cell receptor rearrangements by PCR.^{16,17} Although it took years to gain acceptance, this technique now is a standard tool for hematopathologists. Although the HUMARA assay method for detection of clonality is different, the principle is the same. Similarly, it appears that once sufficiently developed, this assay could have similar value in a wide range of tissues.

As developed in this pilot study, this assay is not sufficiently robust to be used in everyday applications. Further work is underway to increase the percentage of informative cases, decrease the complexity of the assay, and develop in situ applications. Once this test is performed readily, it may be used in many cases in which a definitive morphologic diagnosis is difficult (including application as an ancillary test for the "atypical" breast FNA cases). In the longer term, this test has the potential for automation. Although it remains far from that point, one can conceive of its use, with material obtained by FNA, for nonmorphologic-based diagnoses of clonality.

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Short Communication

Frequent Nuclear/Cytoplasmic Localization of β -catenin without Exon 3 Mutations in Malignant Melanoma

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β -catenin has a critical role in E-cadherin-mediated cell-cell adhesion, and it also functions as a downstream signaling molecule in the *wnt* pathway. Mutations in the putative glycogen synthase kinase 3 β phosphorylation sites near the β -catenin amino terminus have been found in some cancers and cancer cell lines. The mutations render β -catenin resistant to regulation by a complex containing the glycogen synthase kinase 3 β , adenomatous polyposis coli, and axin proteins. As a result, β -catenin accumulates in the cytosol and nucleus and activates T-cell factor/lymphoid enhancing factor transcription factors. Previously, 6 of 27 melanoma cell lines were found to have β -catenin exon 3 mutations affecting the N-terminal phosphorylation sites (Rubinfeld B, Robbins P, Elgamil M, Albert I, Orfiri E, Polakis P: Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* 1997, 275:1790-1792). To assess the role of β -catenin defects in primary melanomas, we undertook immunohistochemical and DNA sequencing studies in 65 melanoma specimens. Nuclear and/or cytoplasmic localization of β -catenin, a potential indicator of *wnt* pathway activation, was seen focally within roughly one third of the tumors, though a clonal somatic mutation in β -catenin was found in only one case (codon 45 Ser \rightarrow Pro). Our findings demonstrate that β -catenin mutations are rare in primary melanoma, in contrast to the situation in melanoma cell lines. Nonetheless, activation of β -catenin, as indicated by its nuclear and/or cytoplasmic localization, appears to be frequent in melanoma, and in some cases, it may reflect focal and

transient activation of the *wnt* pathway within the tumor. (*Am J Pathol* 1999, 154:000-000)

β -catenin is a 92-kd protein, initially identified as a co-precipitating species with the E-cadherin cell-cell adhesive complex.¹ β -catenin was subsequently shown to link E-cadherin to α -catenin, a vinculin-like protein that, in turn, links the E-cadherin/catenin complex to the cortical cytoskeleton.² Molecular cloning revealed that β -catenin is a member of the armadillo (*arm*) family of proteins, whose prototype molecule, Arm, functions as a downstream component of the wingless (*wnt*) signaling pathway in *Drosophila*.³

Although the role of β -catenin in mammalian cells is not completely understood, like the Arm protein, it has been shown to function in the *wnt*-signaling pathway. The present model relating β -catenin to the *wnt* pathway is the following. 1) Binding of the Wnt protein to the Frizzled transmembrane receptor activates the Disheveled protein, which, in turn, inhibits the activity of glycogen synthase kinase 3 β (GSK3 β). 2) GSK3 β , when complexed with the adenomatous polyposis coli (APC) tumor suppressor protein and the axin protein, appears to phosphorylate specific serine and threonine residues in the amino N-terminal region of β -catenin. 3) Phosphorylated β -catenin, but not the unphosphorylated form, is rapidly degraded by the ubiquitin-proteasome pathway. Hence, because *wnt* activation inhibits GSK3 β , *wnt* activation promotes accumulation of unphosphorylated β -catenin in the cell. 4) β -catenin can then complex with members of the T-cell factor (Tcf) or lymphoid enhancer factor (LEF) transcription factor family and activate expression of downstream Tcf/LEF-regulated target genes.⁴

This work is supported by grants from the William and Catherine Weldon Donaghue Foundation for Medical Research, NIH RO-1 GM57604 to D. L. Rimm, the U.S. Army (DAMD17-94-J4366) to D. L. Rimm and E. R. Fearon, and NIH RO-1 CA70097 to E. R. Fearon.

Accepted for publication November 18, 1998.

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Mutational inactivation of the APC gene has been found in the majority of colorectal cancers, and, as might be predicted from the model above, constitutive activation of Tcf/LEF transcription activity is seen in such cases. In a subset of the colorectal cancers lacking APC mutations, mutations in the putative GSK3 β phosphorylation sites of β -catenin result in constitutive activation of Tcf/LEF transcriptional activity.⁵ Similar mutations in β -catenin exon 3 affecting its N-terminal phosphorylation sites have been described in other cancers and cancer cell lines, including melanoma cell lines,⁶ medulloblastoma,⁷ endometrial carcinoma,^{8,9} hepatocellular carcinoma,¹⁰ and prostatic adenocarcinoma.¹¹ In a few cancers, in-frame deletions of the N-terminal region of β -catenin have been detected.¹²

In this work, we sought to determine whether β -catenin signaling was frequently activated in primary melanomas. We determined the subcellular localization of β -catenin in 65 malignant melanoma specimens. In addition, sequencing of β -catenin exon 3 was carried out. Although nuclear and/or cytoplasmic localization occurs in nearly one third of melanomas, only one case was found to have a β -catenin mutation.

Materials and Methods

Specimen Acquisition and Patient Population

All cases of metastatic malignant melanoma accessioned to the cytology or surgical pathology service at Yale-New Haven Hospital from 1994 through 1996 were identified. Tumor samples were obtained through the Critical Technologies Program at the Yale University School of Medicine in accordance with Yale University Human Investigation Committee protocol #8219. Of the 81 metastatic melanoma cases from 1994 to 1996, 65 were evaluable for both the immunostaining and DNA analyses. These 65 specimens represent 18 cytology and 47 surgical specimens. For 12 patients, both surgical and cytology specimens were obtained and studied; however, the surgical and cytology specimens were from independent sites.

Immunostaining Procedures

Standard histological sections were cut from paraffin blocks and prepared for immunostaining using a pressure cooker antigen retrieval method.¹³ In brief, each section was baked at 60°C overnight, then deparaffinized, and treated for antigen retrieval by immersion in 6.5 mmol/L sodium citrate (pH 6.0) for 5 minutes in a conventional pressure cooker (KMart Inc.). Sections were then blocked with 3% bovine serum albumin in Tris-buffered saline (TBS) (150 mmol/L NaCl, 20 mmol/L Tris, pH 8). An anti- β -catenin monoclonal antibody (Transduction Labs, Lexington, KY) was diluted to 2 to 7 μ g/ml and incubated in a humidity chamber overnight, followed by seven TBS washes including 0.01% Triton X-100 in the 6th wash. For increased sensitivity and better subcellular localization of β -catenin, Cy3-conjugated secondary antibodies were used instead of conventional enzymatic

reaction-based chromogens. A Cy3-conjugated goat anti-mouse immunoglobulin antibody (Jackson ImmunoResearch Labs, West Grove, PA) was diluted 1:500 in TBS with 3% bovine serum albumin and incubated with the sections for 1 hour before washing as above and coverslipping. Cytological specimens were prepared for immunohistochemical analysis using the Cytoc (Boxborough, MA) 2000 Thin PrepTM processor. Slides were rinsed for 5 minutes in tap water followed by 5 minutes in TBS (150 mmol/L NaCl, 20 mmol/L Tris, pH 8). Slides were then blocked for 20 minutes with diluted normal serum and washed once in TBS before overlay with the β -catenin monoclonal antibody diluted 1:250 in TBS. After 30 minutes of incubation, slides were subjected to 3 washes with TBS for 5 minutes each. Then 200 μ l of Cy3-Goat anti-mouse antibody (Jackson ImmunoResearch Labs), diluted 1:500 in TBS with 3% bovine serum albumin, was incubated for 30 minutes. Slides were then washed and coverslipped.

DNA Purification

For the cytological specimens, cells in 1 to 20 ~~mls~~ were sedimented to remove the PreservCytTM (Cytoc) and then resuspended in digestion buffer containing 10 mmol/L Tris, pH 8, 100 mmol/L NaCl, 25 mmol/L EDTA, 0.5% sodium dodecyl sulfate, and proteinase K to 100 mg/ml. Each specimen was incubated overnight at 37°C before extraction in an equal volume of phenol/chloroform. DNA was extracted once, precipitated with cold ethanol, washed with 70% ethanol, and resuspended in ~~YE~~ buffer. For the formalin-fixed and paraffin-embedded specimens, DNA was purified from five 20- μ m-thick sections using standard proteinase K and phenol/chloroform extraction methods.

Polymerase Chain Reaction (PCR) Amplification and Sequencing of β -Catenin

PCR was carried out with Taq polymerase on the genomic DNA samples from the cytology and surgical resection specimens using a β -catenin exon 2 forward primer (5'-CGTGGACAATGGCT-ACTCAA-3'), a β -catenin exon 4 reverse primer (5'-TGCATACTGTCCAT-CAATA-3'), and the following conditions: hot start at 95°C, 95°C for 45 seconds, 52°C for 45 seconds, and 72°C for 1 minute. The 700-bp product was purified from agarose gels using the GeneClean Kit (Bio101, Vista, CA). Sequencing of both strands was carried out using Thermo-Sequenase and ³²P-labeled ddNTPs (Amersham Life Science Inc., Arlington Heights, IL) with internal primers (5'-TGGGT-CATATCACATTCTTTT-3' and 5'-CTCT-TACCAGCTACTTGTCTTGA-3').

Results and Discussion

Previous studies have shown that activation of the *wnt* pathway results in an increase in the free cytoplasmic pool of β -catenin and its translocation to the nucleus,

ovarian carcinoma

2

milliliters

Tris/EDTA

e
(should be polymerase)

presumably via the binding of β -catenin to Tcf/LEF family members.¹⁴ Mutations in the APC tumor suppressor gene or the N terminus of β -catenin itself have also been shown to result in increased levels of β -catenin, its localization in the nucleus, and Tcf/LEF transcriptional activation.^{6,15} Hence, we undertook studies to assess the subcellular localization and abundance of β -catenin in 65 melanoma specimens. As an internal control (for the surgical specimens), the localization of β -catenin at the membrane of endothelial cells in capillaries was observed. Membrane localization of β -catenin was seen in a fraction of the neoplastic cells in nearly all specimens. This result was expected because melanocytes have previously been shown to express N-cadherin and other types of cadherin on their cell surfaces,¹⁶ and, as with E-cadherin, β -catenin is bound to the cytoplasmic domain of N-cadherin. Although nearly all specimens showed strong membrane staining, some cases also showed increased cytoplasmic staining and nuclear staining for β -catenin. In this study, the primary goal was to score the neoplastic cells in the melanoma specimens for clear evidence of nuclear and/or cytoplasmic β -catenin staining compared with the membranous pattern seen in normal melanocytes.

We detected nuclear and/or cytoplasmic β -catenin staining in 10 of 18 cytology specimens and 8 of 47 surgical specimens. In the 10 cytology specimens with nuclear β -catenin staining, a high percentage of the cells showed strong staining of the nucleus (Figure 1). In contrast, in the eight surgical specimens with nuclear and/or cytoplasmic β -catenin staining, only 1 to 3% of the nuclei in a given field showed nuclear and/or cytoplasmic staining, whereas the remainder of the neoplastic cells displayed membranous staining for β -catenin. (Figure 2). Table 1 summarizes the staining patterns found. For 12 of the 47 surgical specimens, a cytology specimen from the same patient had also been obtained but from an independent metastatic lesion. Of these, 10 cases showed the same pattern on cytology and surgical specimens, including three cases in which both specimens showed nuclear localization and seven in which both showed membrane staining. In contrast to these 10 cases in which similar staining patterns were seen, in 2 of the 12 cases nuclear and/or cytoplasmic β -catenin staining was seen in the patient's cytology specimen, but only membranous staining was seen in the surgical specimen. The differences in the percentage of cells with nuclear β -catenin between cytology and surgical specimens and the two cases with discordant results on the cytology and surgical specimens are quite curious. The findings may reflect tumor cell heterogeneity among different metastatic deposits within a single patient. An alternative and perhaps more likely explanation is that the consistent increase in the fraction of cells with β -catenin nuclear staining in cytology specimens may be specifically related to specimen preparation. For instance, the mild alcohol-based fixation methods used for the cytology specimens may more readily conserve nuclear β -catenin staining than the formalin fixation used for the surgical specimens.

To assess the prevalence of β -catenin mutations in primary melanomas and to determine whether there was

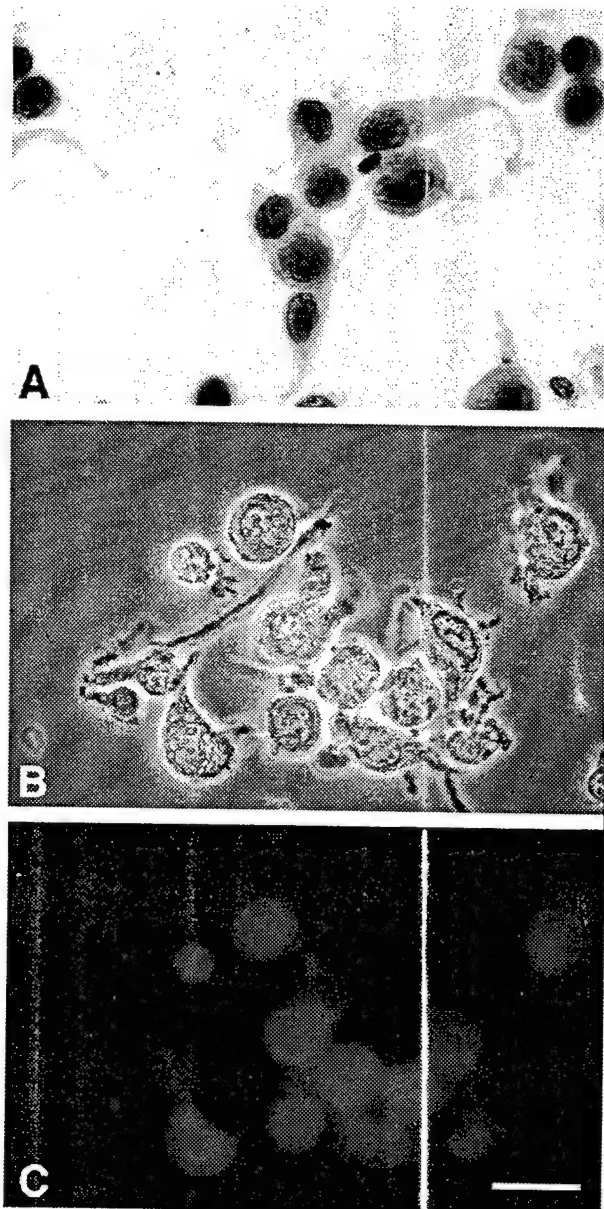


Figure 1. An example of nuclear and/or cytoplasmic localization of β -catenin in a cytology specimen. A: A Thin PrepTM specimen stained with the Papanicolaou stain shows a cluster of melanoma cells aspirated from a metastatic lesion from a malignant melanoma. B: A phase image of another slide of the same cytology specimen. C: The same field viewed with indirect immunofluorescence demonstrates staining of the nuclei with the β -catenin monoclonal antibody and the Cy3-conjugated secondary antibody. Scale bar, 20 μ m.

a strong correlation between nuclear β -catenin staining and β -catenin mutations, we carried out direct sequencing studies of PCR products generated from genomic DNA preparations of the specimens. The cytology specimens used for DNA isolation generally contained greater than 75% neoplastic cells, and the surgical specimens were selected such that the neoplastic cells represented greater than 75% of the total area of the section. Of the 50 specimens for which sufficient DNA was recovered for PCR, only one tumor specimen was found to have a β -catenin mutation. The mutation, a T to C transition at the first position of codon 45, resulted in a change from

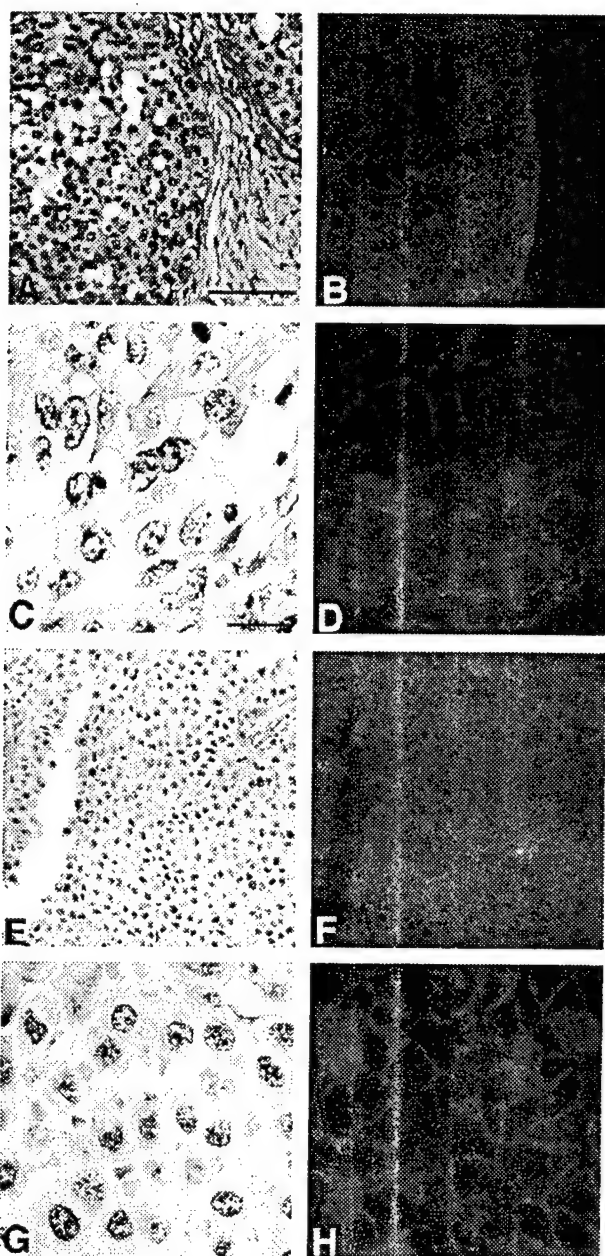


Figure 2. β -catenin localization in surgical specimens shows membrane and scattered nuclear and/or cytoplasmic staining. A, C, E, and G show the hematoxylin and eosin stains of serial sections of the tissues. In B, D, F, and H, an adjacent section from each case has been stained with β -catenin antibody and visualized with the Cy3 fluorophore. H&E-stained slides cannot be studied using immunofluorescence; hence, adjacent sections were studied. Low and high power views are shown of two different cases, one with that demonstrated only membrane staining (A-D) and another that demonstrated nuclear staining (E-H). Original magnification, $\times 200$ (A, B, E, and F); $\times 1000$ (C, D, G, and H). Scale bar, 100 μm (A); 20 μm (C).

serine to proline, and it was confirmed in independent PCR and sequencing reactions. The codon 45 mutation was found in a cytology specimen that showed nuclear staining for β -catenin (Figure 1). This serine to proline mutation has been seen in other studies including two cases of hepatocellular carcinoma,¹⁰ one colonic carcinoma,¹⁷ and (in our group) anaplastic carcinoma of the thyroid (Rostan et al, *in preparation*). In the only other published study of melanoma specimens (cell lines), this serine codon was mutated to tyrosine.⁶

Table 1. β -Catenin Expression and Localization in Metastatic Malignant Melanoma

Tissue type	Nuclear/ cytoplasmic	Membrane	Total
Cytology	10 (55%)	8	18
Surgical	8 (17%)	39	47
Total	18 (28%)	47	65

The distribution of localization of β -catenin expression in the cytology and surgical specimens. Note that nuclear and/or cytoplasmic localization was found in nearly 1/3 of the specimens but with higher frequency in cytology specimens.

In the study of β -catenin mutations in melanoma cell lines, Rubinfeld et al⁶ found 6 of 27 (22%) of the cell lines had mutations affecting exon 3. The frequency of clonal mutations in β -catenin in primary metastatic melanomas appears to be substantially lower with only 1 of 50 (2%) tumors in our study harboring a mutation. The disparate results raise the possibility that β -catenin mutations in many melanoma cell lines may not have been present in primary tumor tissue, but may have arisen during *in vitro* culture. Another possibility is that melanomas with β -catenin mutations may be more readily adapted to *in vitro* culture than melanomas lacking β -catenin mutations. Yet a third possibility is that β -catenin mutations may be present as a subclonal genetic defect in some melanomas, and those neoplastic cells with β -catenin mutations may have more robust growth properties *in vitro* than those cells lacking β -catenin mutations and thus may be more readily grown in culture. Consistent with this proposal, in prostate cancer, β -catenin mutations were seen in only selected regions of primary tumors.¹¹ In addition, in our immunohistochemical studies of melanomas, we found that β -catenin nuclear and/or cytoplasmic localization was a focal and a subclonal alteration in the majority of tumor specimens with nuclear β -catenin staining. Finally, it is also possible that in some cases a mutation was present in only a small minority of cells, and thus it escaped detection in this study.

Overall, nuclear localization of β -catenin was seen in 28% of the 65 metastatic melanoma specimens that we analyzed. In light of the low frequency of β -catenin mutations in our study, the immunohistochemical findings suggest that there may be other mechanisms through which β -catenin may be activated. Indeed, in the majority of colorectal cancers, mutations in the APC gene are responsible for β -catenin activation. Rubinfeld et al⁶ previously found evidence of β -catenin activation as a result of APC inactivation in 2 of the 27 melanoma lines. Hence, APC inactivation may underlie nuclear localization of β -catenin in some of the melanomas that we have studied. Similarly, mutations in GSK3 β , axin, or other elements in the *wnt* pathway may contribute to β -catenin nuclear localization in some tumors. Finally, it is important to emphasize that nuclear localization of β -catenin, particularly the focal and heterogeneous staining patterns that we and others have observed in primary tumors, might even reflect transient and physiological activation of the *wnt* pathway in some cases. As such, at this time, it would be premature to conclude that nuclear and/or

cytoplasmic localization of β -catenin in cancer cells provides definitive evidence of constitutive deregulation of the *wnt* pathway. Nevertheless, our studies establish that β -catenin is mutated in some primary melanomas and suggest that additional studies of the role of *wnt* pathway alterations in melanoma should provide further insights to its pathogenesis.

Acknowledgments

We thank Tom D'Aquila for his expert technical assistance in preparation and staining of tissue and cytology slides.

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Frequent Mutation and Nuclear Localization of β-catenin in Anaplastic Thyroid Carcinoma.

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Running title: β-catenin mutation in Anaplastic Thyroid Carcinoma

Keywords: Cell adhesion, signal transduction, cadherin, APC, Tcf/LEF, Wnt pathway

Footnotes to the title page:

¹This work is supported by grants from the William and Catherine Weldon Donaghue Foundation for Medical Research and NIH RO-1 GM57604 to D.L.R. and the US Army (DAMD17-94-J4366) to D.L.R. and FIS grant (file 97/5063) from the Spanish Government to G.G.R.

²Abbreviations: APC, adenomatous polyposis coli; Tcf, T cell factor; LEF, lymphoid enhancing factor; arm, armadillo; kDa, kilodalton; GSK3β, glycogen synthase kinase 3β; DSH, dishevelled; wnt, wingless; SSCP, single strand conformational polymorphism.

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Abstract

β -catenin is an ubiquitously expressed cytoplasmic protein that has a crucial role in both E-cadherin-mediated cell-cell adhesion and as a downstream signaling molecule in the *wnt* pathway. Stabilization of β -catenin followed by nuclear translocation and subsequent Tcf/LEF mediated transcriptional activation has been proposed as an important step in oncogenesis. Stabilization, may occur through activating mutations in exon-3 at the phosphorylation sites for ubiquitination and degradation of β -catenin.

Immunohistochemical subcellular localization of β -catenin and mutational analysis of exon-3 of the β -catenin gene by SSCP followed by DNA sequencing was performed on 37 samples from 31 patients with anaplastic thyroid carcinoma. Immunofluorescent staining showed nuclear localization in 15/36 samples examined (42%). Nucleotide sequencing of mobility shifts detected by SSCP revealed somatic alterations in 19 of the 31 patients analyzed (61%). We conclude that mutations in β -catenin are common in anaplastic thyroid cancer and that they may activate transcription, as illustrated by frequent nuclear localization of the protein. These findings support the idea that β -catenin acts as an oncogene and contributes to the highly aggressive behavior of this tumor.

Introduction:

β -catenin is a 92 kilodalton (kDa) protein, initially identified as a co-precipitating species with the E-cadherin cell-cell adhesive complex (1). β -catenin was subsequently shown to link E-cadherin to α -catenin which links the E-cadherin/catenin complex to the cortical cytoskeleton (for review see (2)). Molecular cloning revealed that β -catenin is a member of the *armadillo* (*arm*) family of proteins, whose prototype molecule, Arm, functions as a downstream component of the *wingless* (*wnt*) signaling pathway in *Drosophila* (3).

The role of β -catenin in signal transduction is not completely understood, but it has been shown to be involved in the *wnt* developmental pathway. Normally, *wnt* binding to the human *frizzled* homologue activates DSH (dishevelled) signaling protein which inhibits glycogen synthase kinase 3 β (GSK3 β) (4). When the kinase is inactive, it no longer phosphorylates serines and threonines in the N-terminal region of β -catenin (at positions 37-45) and the unphosphorylated form of β -catenin is not degraded by APC in the ubiquitin-mediated proteasome pathway (5,6). Instead, it accumulates in the cytoplasm where it can interact with Tcf-4 (T cell factor 4) or LEF (lymphoid enhancer factor) family (7) of transcriptional activators and result in activation of some developmentally related genes, recently shown to include *c-myc* (8). Mutations in APC that result in its inability to degrade β -catenin or mutations in β -catenin that prevent phosphorylation result in activation of this pathway due to increased cytoplasmic β -catenin (9,10). This is the proposed mechanism of oncogenesis for β -catenin (11).

β -catenin mutations have been reported in both cell lines and human tumors. Initial studies identified mutations at putative GSK3 β phosphorylation sites in colon cancer (9) and melanoma cell lines (10). Shortly thereafter mutations were also found in human colon cancer. Predictably, tumors with APC mutations showed no mutations in β -catenin, while β -catenin mutations were found in nearly 50% of the colon cancers with wild type APC (12). β -catenin mutations have also been detected, at a lower prevalence, in a variety of other cancers including medulloblastoma (13), endometrioid ovarian

carcinoma (14), uterine endometrial carcinoma (15), hepatocellular carcinoma (16) and prostatic adenocarcinoma (17). It has also been found that deletions (as opposed to point mutations) within the N-terminal region could also trigger the oncogenic effect (18).

In this work, we examine for β -catenin mutations anaplastic thyroid carcinoma, a highly lethal neoplasm which is regarded as the final step in the progression of thyroid tumors. We used both SSCP to detect mutations and immunofluorescence to assess nuclear localization of β -catenin. We find that mutations in exon 3, at or near the sites of GSK3 β phosphorylation, are more frequent in this tumor than any cancer examined to date and that there is good, but incomplete correlation between exon 3 mutations and nuclear localization.

Materials and Methods:

Tissue samples: The study population included 37 formalin-fixed paraffin-embedded specimens obtained from 31 patients diagnosed of anaplastic thyroid carcinoma at Hospital Covadonga – University of Oviedo (Spain) and Hospital Yale New Haven – Yale University (USA). For each case, sex and age of the patient at diagnosis, tumor size, extrathyroid extension, vascular invasion, lymph nodes or distant metastasis, stage and follow-up, were recorded. All processing of materials and clinical information proceeded in accordance with Yale University Human Investigation Committee protocol #8219 to D. Rimm.

Fluorescence immunohistochemistry for β -catenin: Representative blocks of tumor tissue were selected on each case to carry out the immunofluorescence analysis of β -catenin. Histologic sections were prepared for immunostaining using a pressure cooker antigen retrieval method (19). Each section was baked at 60° C overnight, then deparaffinized, and treated for antigen retrieval by immersion in 6.5 mM sodium citrate (pH=6.0) for 5 min in a conventional pressure cooker (Kmart Inc.). Sections were then blocked with 3% BSA in Tris-buffered saline (TBS) (150 mM NaCl, 20 mM Tris pH=8). An anti- β -catenin monoclonal antibody (Transduction Labs, Lexington, KY) was diluted to 2-7 μ g/ml and incubated in a humidity chamber overnight, followed by seven TBS washes, including 0.01% triton X-100 in the 6th wash. For increased sensitivity and better subcellular localization of β -catenin, Cy3-conjugated secondary antibodies were used instead of conventional enzymatic reaction-based chromogens. A Cy3-conjugated goat anti-mouse immunoglobulin antibody (Jackson ImmunoResearch Labs, West Grove, PA) was diluted 1:500 in TBS with 3% BSA and incubated with the sections for one hour before washing as above and coverslipping.

DNA Isolation: Genomic DNA was extracted from the same block evaluated for β -catenin expression. Though most of the blocks contained only tumor tissue, some also disclosed normal thyroid follicles entrapped within the tumor. In the latter cases, tumor

material was micro-dissected away from normal tissue for performing the mutational analysis. Embedded sections were deparaffinized using 2 successive washes of xylene, hydrated through 80% ethanol/ 20% xylene solution and vacuum dried. Samples were then incubated overnight in 1M sodium thiocyanate (NaSCN) for protein denaturation. Proteins were digested with proteinase K (10mg/ml) in DNA isolation buffer (5M NaCl, 0.5 M EDTA pH8.0, 0.5% Tween 20, ddH₂O) at 37°C in a shaking water bath overnight. The mixture was then extracted with equal volumes of phenol and phenol: chloroform: Isoamyl-alcohol (25:24:1) in Phase Lock Gel TM tubes (5prime-3prime, Boulder, CO). Nucleic acids were precipitated with 3M sodium acetate (NaAc) in 100% ethanol at -20°C overnight. DNA was pelleted, air-dried and resuspended in 10mM Tris, 1mM EDTA, pH 8.0 (TE).

Mutational analysis: Tumor DNA was evaluated for mutations in the GSK-3 β phosphorylation consensus motif of the β -catenin gene by polymerase chain reaction - single strand conformational polymorphism (PCR-SSCP). DNA sequences of the third coding exon of the β -catenin gene were amplified using the forward 5'-primer GCTGATTTGATGGAGTTGGA and the reverse 3'-primer GCTACTTGTTCTTGAGTGAA. The PCR was carried out in 30 μ l reaction mixture containing 20-100 ng of genomic DNA, 20 pmol of each primer, 250 μ M of each dNTP, 2 mM MgCl₂, 10X Perkin Elmer buffer II (Perkin Elmer Applied Biosystems Division, Foster City, CA) and 2.5 U of AmpliTaq GoldTM (Perkin Elmer). The mixture was heated for 10 minutes at 94°C for initial DNA denaturation, followed by 35 cycles of denaturation (94°C-1 min), annealing (55°C-2min) and extension (72°C-3min), on the GeneAmp PCR system 9600 (Perkin – Elmer Corp.). The PCR products were electrophoresed in a 3% Nusieve: Agarose LE gel (2:1) and visualized by staining with ethidium bromide.

For SSCP analysis , 30ng – 40ng of PCR product were denatured by adding 2.5 to 5 vol. of stop solution (95% formamide, 5.0 M NaOH, 0.1% bromophenol blue and 0.1%

xylene cyanol) and heated at 95°C for 5 min. After quick chill on ice samples were loaded onto a 40% MDE vertical gel (Hydrolink, AT Biochem) and run for 4 hours in a SE 600 vertical gel apparatus (Hoefer Scientific, San Francisco, CA, USA) at 400 Volts at 18°C. Gel temperature was regulated with a cooling circulating bath. After electrophoresis the gels were stained for 10 to 20 min with SYBR – Green II (FMC BioProducts, Rockland, ME) diluted 1:10,000 in TE pH7.4. Images were captured on an IS 1000 Digital Imaging System (Alpha – Innotech Corp., San Leandro, CA, USA) using 254 nm or 313 nm ultraviolet transillumination and a SG-3 filter. All 19 mutations were verified by repeated PCR and SSCP gel analysis.

Sequencing analysis: All samples exhibiting mobility shifts by SSCP were excised and eluted in TE pH 8.0. The eluted DNA was reamplified using the same corresponding set of primers under the same PCR conditions previously described. The products were separated and purified on a 3% Nusieve: Agarose LE gel (2:1) using NA 45 DEAE membranes (Schleicher and Schuell Inc., Keene, N.H.). The DNA bound by the membranes was released in 1.0 M NaCl, 0.1M EDTA, 20mM Tris pH8.0 at 70°C for 10 min and precipitated with 100% ethanol at -20°C overnight. DNA sequences were determined in all cases by dye terminator cycle sequencing using an Applied Biosystem 377 DNA sequencer (Perkin – Elmer Corp). Sequencing from both the sense and antisense orientations was performed for confirmation.

Results and Discussion

Previous studies have shown that activation of the *wnt* pathway results in up-regulation of cytoplasmic β -catenin and its translocation to the nucleus, presumably via the binding of β -catenin to Tcf/LEF family members (20). Thus, as a first assessment, we examined the subcellular localization of β -catenin in 37 anaplastic thyroid carcinoma specimens.

Nuclear immunofluorescence was observed in 15 out of 36 specimens analyzed (41.6%) (Fig. 1) while another 15 specimens were totally negative for membranous, cytoplasmic or nuclear β -catenin staining (41.6%). Other patterns included discontinuous membranous staining (seen in 7 tumor samples (19.4%), 2 of which also showed nuclear staining) and abnormal cytoplasmic localization (seen in 5 samples (13.8%), 3 of which also showed nuclear staining). Normal thyroid follicles entrapped within the tumor, and capillary endothelial cells served as internal positive controls for scoring β -catenin expression in all samples. Negative control sections in all cases showed no staining.

Characteristic mobility shifts were seen in SSCP analysis of exon 3 PCR products (Fig. 2A). Nucleotide sequencing of individual aberrant bands revealed somatic alterations in 19 of the 31 patients analyzed (61.3%). The specific nucleotide changes and the corresponding amino acid substitution are summarized in Table 1 and a sample sequence trace is shown in figure 2B. The potential phosphorylation target residues (serine/threonine) important for β -catenin degradation were mutated in 15/19 patients (78.9%) while tumor samples from the remaining 4 patients were found to be mutated at codons adjacent to, or within a few residues of, the serine or threonine sites (22, 36, 43, 44, 49, 58). These mutations may also affect the formation of the GSK-3 β -APC-axin complex and its ability to phosphorylate and degrade free β -catenin. Similarly to previous analysis of uterine endometrial carcinoma (15), the most common type of mutation affecting putative serine or threonine phosphorylation sites was a C:G>T:A transition present in 11 out of 15 patients. This type of spontaneous mutation affecting C:G dinucleotides has been associated to endogenous oxidative deamination of the exocyclic amino group at 5-methylcytosine leading to replacement of cytosine by thymine, which

is not readily recognized by repair enzymes resulting in C:G>T:A transition (21). Among the 19 mutated patients the most frequent alteration was a Lys to Arg (A:T>G:C) transition at codon 49 seen in 9 patients. Mutations at Ser 33, Ser 37, Thr 41 and Ser 45 reported in the original analysis of β -catenin as oncogene (9,10) were all seen in this cohort. Although a number of studies have now described mutations corresponding to aminoacid residues neighboring the phosphorylation sites (16,17), mutations to non-phosphorylatable amino acids exceed those found in serine or threonine (27 to 20) in this study. Figure 3 shows a schematic summary of the location of all the mutations we found in exon 3.

β -catenin nuclear localization was demonstrated in about half of the cases (10 of 19) with mutations in exon 3. Notably, nuclear localization was seen in all the cases with mutations at either Ser 33, Ser 37 or Ser 45, previously reported targets for GSK3 β phosphorylation (9,10). Four patients without mutations in the GSK3 β phosphorylation consensus motif of the β -catenin gene exhibited also nuclear accumulation of the protein. Nuclear distribution on these 4 patients might be due to increased epidermal growth factor-mediated tyrosine phosphorylation of β -catenin and subsequently disruption of the catenin-cadherin complexes (22), to APC inactivation (9) or to molecular alterations in other genes involved in this pathway (*wnt1* oncogene) (23). Twelve of the 17 patients that did not show nuclear staining showed no detectable expression of β -catenin at all (70.5%), possibly due to mutations outside exon 3 which resulted in truncation of the protein or to other alterations leading to β -catenin downregulation.

Our data demonstrate that anaplastic thyroid carcinoma has the highest frequency of mutations in the CTNNB1 coding region of β -catenin reported to date. In keeping with this high level of mutations, many cases showed multiple activating point mutations (14/19) which indicate a remarkable level of genetic instability in this tumor type. The finding of multiple activating point mutations has not been previously reported in

human tumors screened for β -catenin mutations. However, multiple mutations on the same gene (*Ki-ras*) have been previously described in colon carcinoma and have been shown to correlate with advanced clinical stage (24). Our observation of multiple mutations is consistent with the fact that anaplastic carcinoma is, as the name implies an undifferentiated, highly aggressive tumor which represents the final step in the development and progression of thyroid tumors. Moreover, the analysis of multiple samples from tumors exhibiting, in addition to the anaplastic carcinoma, a concurrent better differentiated component (Follicular carcinoma - specimen 104^b associated with Anaplastic carcinoma – specimen 105^b and Papillary carcinoma – specimen 107^c associated with Anaplastic carcinoma – specimen 106^c) indicated that β -catenin mutations are a late event during thyroid tumor progression since mutations were only detected in the anaplastic carcinoma but not in the better differentiated component (see Table 1). On the other hand, analysis of multiple areas with different microscopic appearance (e.g. spindle or giant cell morphology), but same histologic grade within the same tumor (samples 109^d and 110^d; samples 113^e and 114^e; samples 120^f and 121^f) demonstrated similar to a recent study of prostatic adenocarcinoma (17) that β -catenin mutations may be focal (sample 113^e resulted wild type and sample 114^e resulted mutated) and also that anaplastic thyroid carcinoma exhibits clonal heterogeneity (See samples 109^d and 110^d and samples 120^f and 121^f on Table 1). Finally this series also showed for the first time a relatively high rate of silent mutations. Nine out of the 19 mutated patients featured at least one silent mutation, with some cases exhibiting multiple third position replacements. However, we can not exclude the possibility that some of these third base changes represent unreported β -catenin polymorphisms.

In conclusion β -catenin mutations are very common in anaplastic thyroid carcinoma and they may activate transcription, as illustrated by frequent nuclear localization of the protein. Our findings indicate that β -catenin acts as an oncogene in thyroid tumors and contributes to the highly aggressive behaviour of anaplastic thyroid carcinoma. Since this tumor carries an extremely poor prognosis (all the patients in this study died within one

year of the diagnosis) it is impossible to obtain meaningful survival data and no attempt was made to correlate either nuclear localization or mutation of β -catenin to outcome.

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Table 1: CTNNB1 exon 3 mutations in anaplastic thyroid carcinoma

Sample	Codon mutated	Mutation	Ser/Thr or adjacent or other	Nuclear Staining
15 ^a	40 (Thr⇒Ile) 49 (Lys⇒Arg)	ACT⇒ATT AAA⇒AGA	Thr other	Positive
16	40 (Thr⇒Ile) 49 (Lys⇒Arg)	ACT⇒ATT AAA⇒AGA	Thr other	Negative
17	45 (Ser⇒Pro)	TCT⇒CCT	Ser	Positive
21 ^a	40 (Thr⇒Ile) 46 (Leu⇒Val) 49 (Lys⇒Arg)	ACT⇒ATT CTG⇒GTG AAA⇒AGA	Thr adjacent other	Negative
23 ^a	49 (Lys⇒Arg)	AAA⇒AGA	other	Positive
24	17 (Asp⇒His) 40 (Thr⇒Ile) 49 (Lys⇒Arg)	GAC⇒CAC ACT⇒ATT AAA⇒AGA	other Thr other	Positive
101	47 (Ser⇒Asn)	AGT⇒AAT	Ser	Positive
103 ^a	38 (Gly⇒Asp) 41 (Thr⇒Ser) 49 (Lys⇒Arg)	GGT⇒GAT ACC⇒AGC AAA⇒AGA	other Thr other	Negative
105 ^b	40 (Thr⇒Ile) 49 (Lys⇒Arg)	ACT⇒ATT AAA⇒AGA	Thr other	Negative
106 ^{a,c}	20 (Ala⇒Val) 36 (His⇒Tyr) 44 (Pro⇒Ser)	GCG⇒GTG CAT⇒TAT CCT⇒TCT	other adjacent adjacent	Negative
108	41 (Thr⇒Ile)	ACC⇒ATC	Thr	Negative
109 ^d	40 (Thr⇒Ile) 49 (Lys⇒Arg)	ACT⇒ATT AAA⇒AGA	Thr other	Negative
110 ^{a,d}	37 (Ser⇒Phe) 44 (Pro⇒Ser) 60 (Ser⇒Phe)	TCT⇒TTT CCT⇒TCT TCC⇒TTC	Ser adjacent Ser	Positive
114 ^{a,e}	40 (Thr⇒Ile) 43 (Ala⇒Val) 44 (Pro⇒Ser) 52 (Pro⇒Leu)	ACT⇒ATT GCT⇒GTT CCT⇒TCT CCT⇒CTT	Thr adjacent adjacent other	Negative
115	44 (Pro⇒Ser) 47 (Ser⇒Asn) 55 (Glu⇒Lys)	CCT⇒TCT AGT⇒AAT GAG⇒AAG	Adjacent Ser other	Negative
117 ^a	33 (Ser⇒Phe) 37 (Ser⇒Phe) 42 (Thr⇒Ile)	TCT⇒TTT TCT⇒TTT ACA⇒ATA	Ser Ser Thr	Positive
119	40 (Thr⇒Ile) 49 (Lys⇒Arg)	ACT⇒ATT AAA⇒AGA	Thr other	Negative
120 ^{a,f}	22 (Val⇒Ala)	GTT⇒GCT	adjacent	Positive
121 ^{a,f}	55 (Glu⇒Lys)	GAG⇒AAG	other	Positive
122	58 (Asp⇒Asn)	GAT⇒AAT	adjacent	Positive
125	37 (Ser⇒Phe) 45 (Ser⇒Pro) 52 (Pro⇒Leu) 54 (Glu⇒Lys)	TCT⇒TTT TCT⇒CCT CCT⇒CTT GAA⇒AAA	Ser Ser other other	Positive

Table 1 footnotes: Summary of mutations found in the CTNNB1 coding region of the β -catenin gene by specimen number. Standard three-letter code is used to indicate amino acids. "Adjacent" indicates mutation neighboring a serine or threonine residue.

^a indicates silent mutation(s) were also present in this case.

b, c, d, e, f indicate that multiple foci with different morphological patterns or histologic grade were analyzed within the same tumor.

Figure Legends:

Figure 1. Examples of nuclear localization of β -catenin in anaplastic thyroid tumors. Frame (A) shows a 20X objective view of the hematoxylin and eosin (H&E) stained tumor and a serial section (B) stained with an anti- β -catenin monoclonal antibody visualized with a Cy3 conjugated fluorescent secondary antibody. High power (C-F) views illustrate β -catenin immunoreactivity in anaplastic carcinoma and the corresponding H&E and phase contrast images. (C) and (D) are serial sections while the phase image (E) is the identical field and section as that seen stained with β -catenin and visualized with Cy3 in (F). It shows nuclear localization of β -catenin as well as membrane junctional staining of the endothelial cells (annotated by white arrows) which provides the internal positive control for β -catenin expression. Scale Bars: B=100 μ M, D=20 μ M.

Figure 2. (A) SSCP analysis of the PCR amplification of β -catenin exon 3 in six anaplastic thyroid carcinomas. Lane WT shows the wild type band pattern and beneath each of the other lanes are the case numbers of the thyroid tumors. Lower case letters a-g indicate mobility shifts representing somatic mutations at the following codons: a=40, b=40 and 49, c=32, d=44, e=54, f=37 and g=45. The corresponding sequence changes and amino acid substitutions are shown in table 1. (B) The sense sequence of case 125 showing 2 point mutations at serines 37 and 45.

Figure 3. A schematic illustrates the location of the 45 mutations identified in this cohort in exon 3. The standard single letter amino acid code is used. Letters in boldface type indicate cases with nuclear localization of β -catenin.

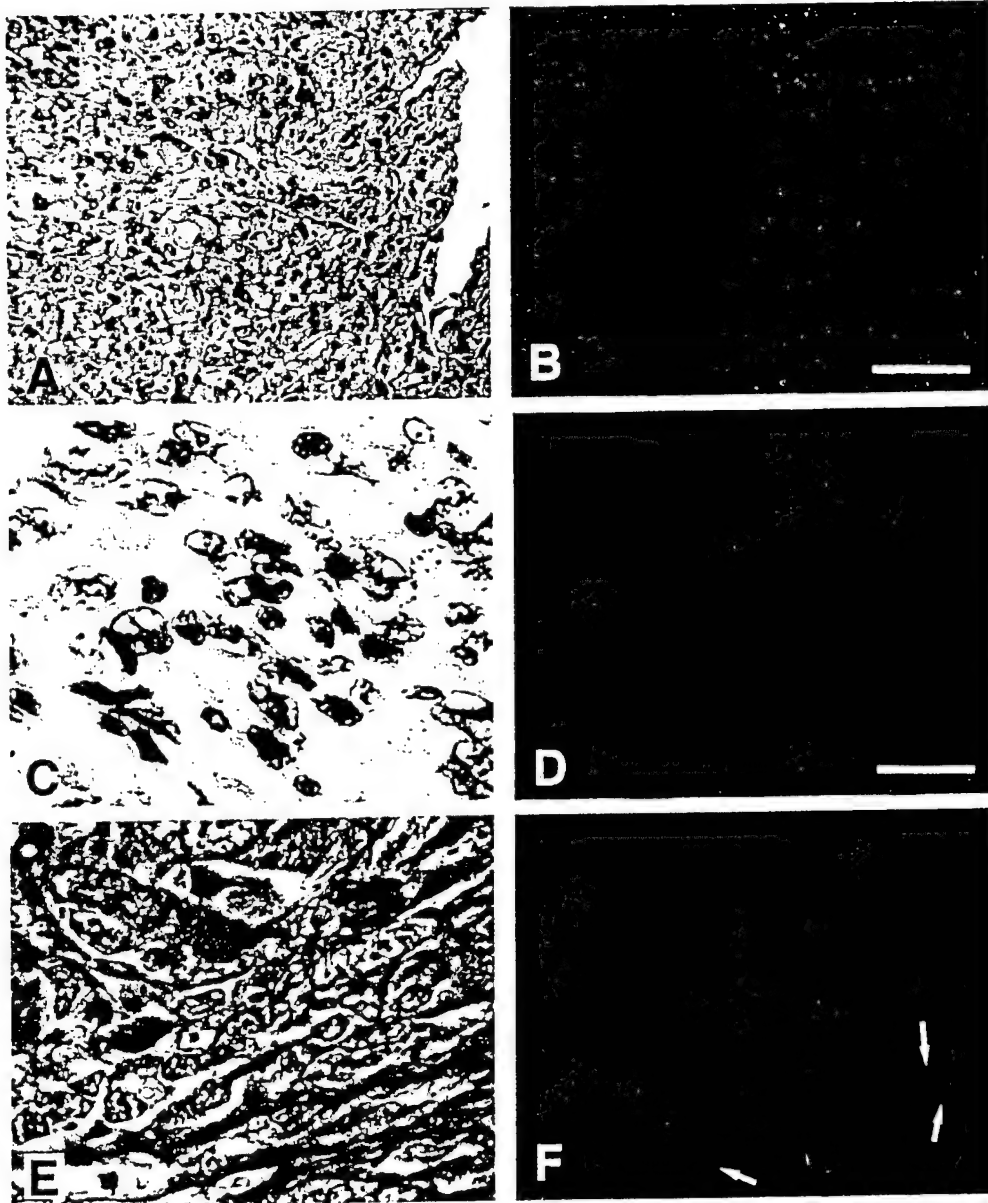


Figure 1
Garcia-Rostan et al, 1998

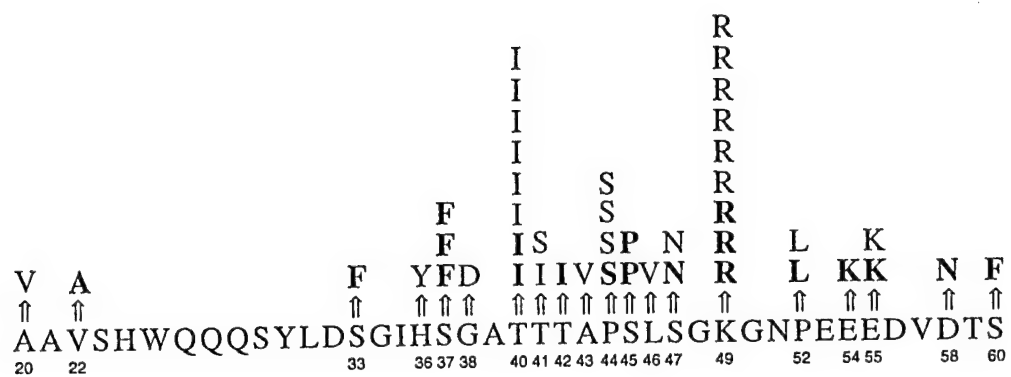


Figure 3
Garcia-Rostan et al, 1998

Garcia-Rostan et al, 1998

β - and γ -catenin Mutations, But Not E-cadherin Alterations, Underlie Tcf/Lef Transcriptional Deregulation in Gastric and Pancreatic Cancer¹

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Running Title: Tcf deregulation in gastric and pancreatic cancer

Keywords: gastric cancer; pancreatic cancer; β -catenin; γ -catenin; Tcf; Wnt pathway; transcription factors;

Abbreviations: APC, adenomatous polyposis coli, β -cat, β -catenin; γ -cat, γ -catenin; E-cad, E-cadherin; Tcf, T cell factor; Lef, lymphoid enhancer factor; CTTA, constitutive Tcf transcriptional activity; GSK3 β , glycogen synthase kinase 3 β ; *CTNNB1* - β -cat gene; *CTNNG1* - γ -cat gene; *CDH1* - E-cad gene

¹This work was supported by grants from the William and Catherine Weldon Donaghe Foundation for Medical Research and NIH GM57604, CA70097; and U.S. Army DAMD17-94-J4366; Deutsche Forschungsgemeinschaft CA207/1 and KO1826/1.

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ABSTRACT

Adenomatous polyposis coli (*APC*) mutations are present in >70% of colorectal cancers, but are rare in other cancers. The *APC* protein binds to β -catenin (β -cat), a protein initially identified because of its role in E-cadherin (E-cad) cell-cell adhesion. In some colorectal cancers lacking *APC* defects, mutations have been found in potential phosphorylation sites near the amino (N)-terminus of β -cat, presumably rendering β -cat resistant to regulation by a complex containing *APC* and GSK3 β . In cells with mutations in the *APC* or β -cat (*CTNNB1*) genes, β -cat is stabilized, in turn, binding to and activating Tcf/Lef transcription factors. To further explore the role of the *APC*/ β -cat/Tcf/E-cad defects in gastrointestinal cancers, we assessed gastric and pancreatic cancers for constitutive Tcf transcriptional activity (CTTA). Two of 4 gastric and 2 of 8 pancreatic cancer lines showed CTTA. One gastric and one pancreatic cancer had mutations in the N-terminal phosphorylation sites of β -cat. The other gastric cancer with CTTA had a missense mutation at serine 28 of γ -cat, a potential phosphorylation site in this β -cat-related protein. In one pancreatic cancer with CTTA, no mutations in the *APC*, *CTNNB1*, or γ -cat (*CTNNG1*) genes were found, suggesting other defects may underlie CTTA. Though E-cad is an important binding partner for β - and γ -cat, E-cad inactivation did not result in CTTA. The β - and γ -cat mutant proteins identified in our studies activated Tcf transcription *in vitro*, while β -cat mutant proteins with larger N-terminal deletions had only modest effects on Tcf activation. Our results indicate a role for Tcf deregulation in gastric and pancreatic cancer, resulting from β - and γ -cat mutations in some cases, and, in others, from yet to be defined defects. Furthermore, the data imply that the role of mutations in *APC* and β -cat in cancer development are distinct from the consequences of E-cad inactivation.

INTRODUCTION

Germline, inactivating mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene are responsible for familial polyposis, and somatic *APC* mutations are present in roughly 70% of colorectal adenomas and carcinomas, but are rare in other cancers (reviewed in Ref. 1). The *APC* protein has been found to bind to a number of different proteins, including β -catenin (β -cat) (2,3), γ -catenin (γ -cat) (also known as plakoglobin) (4,5), glycogen synthase kinase 3 β (GSK3 β) (6), EB1 (7), hDLG (8), and microtubules (9,10). With the exception of β -cat, the significance and role of *APC*'s interactions with its various binding partners remains poorly understood.

β -cat, initially identified as co-precipitating protein with E-cadherin (E-cad), was subsequently shown to link E-cad to α -cat, a vinculin-like protein which, in turn, links the E-cad/cat cell-cell adhesive complex to the cortical cytoskeleton (11-15). Molecular cloning revealed that β -cat is a member of the *armadillo* (*arm*) family, and the prototype molecule Arm functions in the *wingless* (*wnt*) signaling pathway in *Drosophila* (12,15). The γ -cat protein is highly related to β -cat and appears to have similar functions in cell-cell adhesion and the *wnt* pathway (12-15).

The identification of T cell factor (Tcf)/lymphoid enhancer factor (Lef) transcriptional factor proteins as downstream targets of β -cat (16-18), together with the observation of constitutive Tcf transcriptional activity (CTTA) in colorectal cancer cell lines with *APC* defects (19), buttressed the proposal that *APC* had a critical role in regulating β -cat (20). Further support for this proposal was provided by data indicating that a subset of the colorectal cancers lacking *APC* mutations had CTTA as a result of mutations altering potential GSK3 β phosphorylation sites in the amino (N)-terminus of β -cat (21). Subsequent studies have reported mutations affecting the N-terminal phosphorylation sites of β -cat in melanoma cell lines (22), medulloblastoma (23), ovarian carcinoma (24), endometrial carcinoma (25), hepatocellular carcinoma (26) and prostatic adenocarcinoma (27). The presumed consequence of these mutations is that the β -cat protein is

rendered oncogenic as a result of its resistance to regulation by a protein complex containing APC, GSK3 β , and axin (28-30). β -cat accumulates in the cell and deregulates expression of downstream Tcf/LEF-regulated target genes, including perhaps c-myc (31), via its role as a transcriptional co-activator.

As noted above, in addition to their role in wnt signaling, β -cat and γ -cat are key components in E-cad cell-cell adhesion, a function which is critical in development, cell differentiation and maintenance of tissue architecture (11-14). In many epithelial cancer types, loss of E-cad expression has been frequently observed (14). The mechanisms underlying loss of E-cad are rather poorly understood in the majority of cancers with altered expression. However, in some cancers, including diffuse type gastric carcinoma, somatic mutations in the E-cad (*CDH1*) gene underlie loss of its expression (14,32,33). *CDH1* germline mutations have recently been found in several families with inherited predisposition to gastric cancer (34,35), further highlighting the significance of E-cad alterations in gastrointestinal (GI) cancer.

In contrast to the extensive body of data in colorectal cancer, there have been only limited reports of *APC* mutations and no reports of *CTNNB1* or *CTNNG1* mutations in tumors of the upper GI tract or pancreas (36-40), although decreased and/or heterogenous expression of E-cad and/or β -cat have been reported to correlate with high grade or advanced stage tumors in esophageal, gastric, and pancreatic cancer (41-44). To explore further the role of the APC/ β -cat/Tcf and E-cad/cat pathways in cancers of the GI-tract, we examined gastric and pancreatic cancer cell lines for CTTA, using Tcf reporter gene constructs. Our studies indicate a role for Tcf deregulation in gastric and pancreatic cancer, resulting from *CTNNB1* or *CTNNG1* mutations in some cases, and in others from yet to be defined defects. Our findings also imply that the consequences of *APC* or catenin mutations in cancer development are likely to be distinct from those attributable to E-cad inactivation.

MATERIALS AND METHODS

Cell lines. All cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in the recommended growth conditions at 37 °C. Genomic DNA was isolated by incubating cell pellets for 48 hr at 48°C with 0.5 mg/ml proteinase K in 1.0% SDS, followed by phenol-chloroform extractions, and ethanol precipitation. Total RNA was isolated from cells using the RNeasy RNA isolation system (Qiagen, Madison, WI), and protein lysates were prepared in RIPA buffer with protease inhibitors.

DNA transfections and reporter assays. Transfections to assess Tcf transcriptional activation were performed on cells growing at approximately 70% confluence in six-well plates, using 1 ml of Opti-MEM reduced serum medium (Gibco BRL Life Technologies Inc., Grand Island, NY), 6 µl Lipofectin reagent (Gibco BRL), 0.75 µg pcH110, and 0.75 µg of pTopflash (optimal motif) or pFopflash (mutant motif) in each well (21). To assess the effect of wildtype and mutated forms of β - and γ -cat on Tcf-transcriptional activity, co-transfections were undertaken as described above, except that 0.5 µg pcH110; 0.5 µg of pTopflash or pFopflash, and 0.5 µg of the respective β - or γ -cat expression vector were used. To assess the effect of APC on Tcf activity, cells were co-transfected with 0.5 µg pcH110, 0.5 µg of pTopflash or pFopflash, and 0.25 or 0.75 µg of the wildtype APC expression construct. Varying amounts of an empty control vector (pcDNA3) were used to normalize the DNA mass to 2 µg in each transfection. Cell extracts were prepared 24-30 hr post-transfection using reporter lysis buffer (Promega). Luciferase and β -galactosidase assays were carried out as recommended by the manufacturer (Promega). Luciferase activities were measured in a luminometer (model TD-20E, Turner Corp., Mountain View, CA). All transfections were repeated three or more times. For each cell line, the mean luciferase activity of the pFopflash reporter construct was assigned a value of 1, and the luciferase activity with pTopflash was reported relative to the pFopflash activity.

β - and γ -cat mutation analyses. For the 4 gastric and 8 pancreatic cancer cell lines, first strand cDNA was prepared from total RNA, using AMV reverse transcriptase (Promega) and

random hexamers. The entire open reading frames of *CTNNB1* and *CTNNG1* were then amplified from cDNA by PCR with Pfu (Stratagene, La Jolla, CA) and primers β CATFL-S and β CATFL-A and PLAKFL-S and PLAKFL-A, respectively (see Table 1 for primer sequences). In 36 primary gastric cancer specimens, DNA was isolated by microdissection of regions of carcinoma from thick, unstained sections of formalin-fixed and paraffin-embedded tumors. To amplify a *CTNNB1* genomic DNA fragment containing exon 2-4 (codons 1 to 86), PCR was carried out with Pfu, using oligonucleotide primers β CATEX2-S, β CATEX4-A. Similarly, PCR with Pfu was carried out to amplify a genomic DNA fragment containing codons 7-70 of *CTNNG1*, using primers PLAKG-S and PLAKG-A. PCR products were purified following agarose gel electrophoresis and sequenced directly with the following primers: β CATEX2-S and β CATEX4-A for β -cat RT-PCR products; β CATGS-S and β CATGS-A for *CTNNB1* genomic DNA products; and PLAKG-S and PLAKG-A for *CTNNG1* RT-PCR and genomic DNA products. Thermo-Sequenase and ^{33}P -labeled ddNTPs were used according to the manufacturer's instructions (Amersham Life Science Inc., Arlington Heights, IL). Mutations were verified in independent PCR and sequencing studies.

Plasmid constructs. The vector pcDNA3 (Invitrogen, San Diego, CA) was used to generate β - and γ -cat expression constructs. Full-length wildtype *CTNNB1* and *CTNNG1* cDNAs were amplified by PCR from a normal colon cDNA library (Clontech Laboratories Inc., Palo Alto, CA), using primers β CATFL-S and β CATFL-A for *CTNNB1* and PLAKFL-S and PLAKFL-A for *CTNNG1* (Table 1). The oligonucleotides were modified to yield BamHI (5'-end) and XbaI (3'-end) recognition sites at the ends of the *CTNNB1* cDNA, and KpnI (5'-end) and EcoRI (3'-end) sites for the *CTNNG1* cDNA. Mutant *CTNNB1* and *CTNNG1* cDNAs were amplified by PCR from cDNA of selected gastric and pancreatic cancer cell lines with the respective primer pairs. To generate the β -cat Δ N90 and exon 3 deletion constructs, the forward primers β CAT Δ N90-S and β CAT Δ EX3-S were used together with the β CATFL-A reverse primer. PCR amplification of *CTNNB1* and *CTNNG1* cDNAs was carried out with Pfu polymerase, and the sequences of all

PCR products was verified by manual or automated DNA sequencing. The pCH110 eukaryotic expression vector (Pharmacia, Piscataway, NJ), containing a functional LacZ gene cloned downstream of a cytomegalovirus (CMV) early region promoter/enhancer element was used to control for transfection efficiency in the luciferase assays.

Western blot analysis. Approximately 40 μ g of each protein lysate in RIPA buffer was separated by electrophoresis on SDS/polyacrylamide gels and transferred to Immobilon P membranes (Millipore, Bedford, MA) by semi-dry electroblotting (Transblot, Bio-Rad, Hercules, CA). Enhanced chemiluminescence (ECL) Western blot analysis was carried out with the mouse monoclonal antibodies against α -cat, β -cat, γ -cat, GSK-3 β (all from Transduction Laboratories, Lexington, KY) and E-cad (Zymed Laboratories, Inc., San Francisco, CA). A polyclonal antibody against Na⁺/K⁺ ATPase (Research Diagnostics, Inc, Flanders, NJ) was used to verify equal loading of Western blots. Horseradish peroxidase (HRP)-conjugated goat-anti mouse IgG or goat-anti rabbit immunoglobulin antibodies (Pierce Biochemicals, Rockford, IL) were used. Antibody complexes were detected with the ECL-Western blot kit (Amersham) and exposure to X-OMAT film (Kodak, Rochester, NY)

Half-life studies of γ -cat. Chinese hamster ovary (CHO) cells were transfected with a 1.0 μ g of a pcDNA3 construct encoding wildtype or mutant γ -cat, using LipofectAMINE (Gibco BRL). Forty-eight hr after transfection, cells were incubated for 1 hr in methionine-free medium, followed by a 1 hr incubation with 150 μ Ci ³⁵S-methionine (pulse). The cells were washed and incubated in fresh medium (chase). At specific time points, cells were harvested in TBS/1% TritonX-100 lysis buffer containing protease inhibitors. Immunoprecipitation was carried out with a monoclonal anti- γ -cat antibody (Transduction Laboratories and protein A agarose (Pierce) for 2 hr. The immune complexes were recovered, washed, boiled in Laemmli sample buffer, and resolved by SDS/PAGE. Fluorography with Amplify (Amersham) was carried out, and the autoradiographic signals of the γ -cat bands were quantitated by densitometry (Alpha Innotech, Corp, San Leandro, CA).

RESULTS

Constitutive Tcf transcriptional activity (CTTA) in gastric and pancreatic cancer

As reviewed above, previous studies revealed CTТА in colorectal cancer lines with inactivating mutations in *APC*, such as the SW480 cell line, and in lines with missense mutations or deletions of single amino acids of the potential GSK3 β phosphorylation sites at the N-terminus of β -cat, such as the HCT-116 line (19,21). Hence, we assessed Tcf transcriptional activity as an initial strategy for detecting alterations in the regulation or function of APC, β -cat, or Tcf/Lef family members. Two reporter gene constructs, pTopflash and pFopflash, employed in prior studies (19,21), were also used for our studies. The pTopflash construct contains consensus Tcf binding sites cloned upstream of a minimal promoter element and luciferase reporter gene, and pFopflash contains mutated Tcf binding sites cloned upstream of luciferase. In any given cell line, comparison of the luciferase activity of the pTopflash construct versus that of the control pFopflash construct proves a measure of the relative Tcf activity. As expected, the SW480 and HCT-116 colorectal cancer lines showed clear evidence of CTТА (Fig. 1). We then assessed Tcf transcriptional activity in 4 gastric and 8 pancreatic cancer lines. The majority of the lines displayed no evidence of CTТА, with mean relative Tcf activities between 0.25 and 2.0. However, CTТА was found in two gastric (NCI-N87 and AGS) and two pancreatic (ASPC and HS766T) lines, with mean relative activities ranging from about 5 in the NCI-N87 line to 50 in the ASPC line (Fig. 1). The specific Tcf/Lef factor(s) responsible for the observed Tcf reporter gene activity is not known. However, in RT-PCR assays, roughly equivalent levels of hTcf-4 gene expression were found in all lines studied in Figure 1, including the lines lacking CTТА.

β - and γ -cat mutations in gastric and pancreatic cancers

We sought to determine the basis for CTТА in the gastric and pancreatic cancers. Prior findings indicate that *APC* mutations are rare or absent in gastric and pancreatic cancer, and we failed to find evidence of APC inactivation in the cell lines through Western blot studies of APC (data not shown). Because of the contribution of *CTNNB1* mutations to CTТА in a subset of

colorectal cancers, we carried out RT-PCR studies to determine if *CTNNB1* mutations were present in any of the 12 gastric and pancreatic cancer lines. The entire open reading frame of β -cat was amplified, and no evidence of large deletions or insertions was found. Analysis of the sequences encoding the N-terminal region of β -cat (corresponding to codons 1-86) revealed that the AGS gastric cancer cell line had a missense mutation of glycine to glutamic acid at codon-34, and the HS766T pancreatic cancer cell line had a in-frame deletion of serine codon 45 (Fig. 2). Though *CTNNG1* mutations have not been previously reported in cancer, given the very high sequence conservation between the γ -cat and β -cat proteins, we analyzed the sequences encoding the N-terminal region of γ -cat in all 12 of the gastric and pancreatic cell lines. In NCI-N87, we found a missense mutation at *CTNNG1* codon 28, resulting in a serine to leucine substitution and altering a potential phosphorylation site (Fig. 2). No *CTNNB1* or *CTNNG1* mutations were found in ASPC or the 8 lines that failed to manifest CTTA. Sequencing studies of exons 2-4 of *CTNNB1* and the corresponding region of *CTNNG1* in 36 primary gastric cancers, using genomic DNA microdissected from regions enriched in neoplastic cells, failed to identify any *CTNNB1* or *CTNNG1* mutations, suggesting that mutations in β - and γ -cat are likely to be present in only a minority of gastric cancers.

Tcf Transcriptional Activity in the ASPC Line is Not Responsive to APC

Our sequencing-based studies did not identify *CTNNB1* or *CTNNG1* mutations in the ASPC pancreatic cancer line, and our Western blot studies failed to provide evidence of APC inactivation. To address the possibility that the ASPC line might have a subtle mutation in *APC*, we sought to determine if Tcf transcriptional activity in ASPC was inhibited by overexpression of wildtype APC. As expected, overexpression of wildtype APC in colorectal cancer cells lacking APC function, such as the SW480 line, suppressed Tcf transcriptional activity (Fig. 3). Tcf activity was unaffected by APC in colorectal cancer cells with a mutant *CTNNB1* allele, such as HCT-116 cells (Fig. 3). Though Tcf transcriptional activity was only subtly elevated in ASPC, the fact that Tcf activity could not be suppressed by overexpression of wildtype APC implies that CTTA in ASPC

is not attributable to *APC* inactivation. Therefore, defects besides those in the *APC*, *CTNNB1*, or *CTNNG1* genes, are associated with CTTA in some cancers.

Mutant β - and γ -cat Proteins Activate Tcf Transcriptional Activity

Mutant β -cat proteins with missense mutations or in-frame deletions of single amino acids in the N-terminal phosphorylation sites have previously been shown to strongly activate Tcf transcriptional activity when expressed in heterologous cell types, such as the 293 kidney cell line (21). We sought to confirm that the mutant *CTNNB1* and *CTNNG1* alleles that we had identified did, in fact, encode proteins that activated Tcf transcriptional activity. Consistent with prior results, a mutant *CTNNB1* allele encoding a β -cat protein with the codon 33 serine to tyrosine substitution found in the SW48 colorectal cancer line strongly activated Tcf-mediated transcription in 293 cells, while wildtype β -cat had readily detectable but more modest effects (Fig. 3). We found an in-frame deletion of serine codon 45 (Δ S45) in the HS766T pancreatic line, and this same mutation had previously been found in the HCT-116 colorectal line. The Δ S45 mutant *CTNNB1* allele strongly activated Tcf transcription, as did the G33E mutant allele in the AGS gastric cancer line (Fig. 4).

In contrast to the strong activity of these selected mutant *CTNNB1* alleles, the mutant *CTNNG1* allele in the NCI-N87 gastric line (serine to leucine at codon 28 - S28L) had only a modest ability to activate Tcf transcription, though the mutant γ -cat protein was about two-fold more potent than wildtype γ -cat (Fig. 4). The differing strength of the mutant β - and γ -cat proteins in our assay may reflect true differences in their in vivo function, or it may be attributable, at least in part, to the heterologous 293 cell line used for the assay. Nevertheless, the data demonstrating that the S28L mutant *CTNNG1* allele had less activity than the *CTNNB1* alleles with point mutations and small deletions (i.e., S33Y, Δ S45, and G34E) are consistent with the fact that the NCI-N87 line (i.e., the line from which the allele was derived) had less CTTA relative to the lines with mutant β -cat alleles (see Fig. 1). Similar to β -cat proteins with mutations in the N-terminal

phosphorylation sites (22), the γ -cat S28L mutant protein had a prolonged half-life. We found that the half life of wildtype γ -cat was roughly 0.5 hr in Chinese hamster ovary (CHO) cells, while the half-life of the S28L mutant was greater than 2 hr (data not shown).

In addition to missense mutations and single amino acid deletions in the N-terminal phosphorylation sites of β -cat, larger in-frame deletions of β -cat N-terminal sequences have been reported in a few cancer cell lines and primary tumors (22,26,45). The deletions are presumed to promote cancer growth through the same mechanisms as do mutant β -cat alleles with localized mutations in the N-terminus. However, we found that mutant β -cat alleles with large N-terminal deletions, such as deletion of β -cat amino acids 1-90 ($\Delta 90$) or deletion of amino acids 5-80 ($\Delta ex3$), had activities in the Tcf assay indistinguishable from those of wildtype β -cat (Fig. 4). At least two explanations can be considered. First, it is entirely possible that the *in vitro* assay of Tcf transcriptional activity does not accurately reflect the *in vivo* activity of all mutant β -cat proteins. A second explanation is that the assay does, in fact, accurately reflect the ability of mutant β -cat proteins to activate Tcf transcription, but β -cat proteins with larger N-terminal deletions promote cancer development through mechanisms largely independent of Tcf activation.

Loss of E-cad Expression Does Not Lead to CTTA

In normal cells, β -cat is a highly abundant protein, complexed primarily with E-cad at the plasma membrane. As reviewed above, E-cad expression is often lost in many epithelial cancers, including gastric cancer. To examine the possibility that E-cad inactivation might lead to increased levels of β -cat in the cell and ultimately CTTA, we first examined the expression of E-cad, and then that of α -, β -, and γ -cat and GSK3 β in the 4 gastric and 8 pancreatic cancer cell lines. Three of the 4 gastric cancer lines and one pancreatic cancer line had very reduced or absent E-cad expression (Fig. 5). Comparison of the pattern of E-cad expression in the lines (Fig. 5) to their Tcf transcriptional activity (Fig. 1) revealed no clear association. The abundance of β - and γ -cat was minimally increased in the 2 gastric cancer lines with β - or γ -cat mutations (AGS has a β -cat

mutation and NCI-N87 has a γ -cat mutation) (Fig. 5). Our failure to find a strong correlation between *CTNNB1* and *CTNNG1* mutations and the relative abundance of the respective protein products in the lines may be largely attributable to the fact that we assessed total levels of β - and γ -cat in the cells, rather than "free" cytosolic pools. All cell lines expressed roughly equivalent levels of GSK3 β (Fig. 5).

We also assessed Tcf transcriptional activity in 8 breast cancer lines, including 5 lines lacking E-cad expression. No evidence for CTTA was found in any of the lines (data not shown). To determine if the signaling pathways for responding to deregulated β - or γ -cat proteins were intact in breast cancer, we assessed the ability of wildtype and mutated forms of β - and γ -cat to activate Tcf transcription in BT549 breast cancer cells, a line lacking endogenous E-cad expression. As shown in Fig. 6, mutated β - and γ -cat proteins were able to stimulate Tcf transcriptional activation more potently than their wildtype counterparts. The findings indicate that Tcf/Lef family members are expressed in breast cancer cells, but deregulation of Tcf transcription appears to be a rare event in breast cancer. Furthermore, the data from our breast cancer studies support the proposal that, in contrast to mutations in the *APC*, *CTNNB1*, or *CTNNG1* genes, E-cad inactivation does not lead to Tcf transcriptional deregulation.

DISCUSSION

Recent observations have greatly advanced our understanding of the significance of the interaction between the APC tumor suppressor protein and β -cat. Together with other proteins, such as the GSK3 β kinase and axin, APC functions to regulate the stability and abundance of β -cat in the cytosol, presumably in response to signals from upstream pathways, such as those from the Wnt signaling pathway. If β -cat accumulates in the cytosol, it can bind to Tcf/Lef transcription factors, and following translocation to the nucleus, the Tcf/ β -cat complex activates expression of cellular genes, perhaps including c-myc (31). In colorectal cancers, *APC* inactivation has been seen in upwards of 70-75% of cases, and a subset of the cancers lacking *APC* mutations, harbor

activating mutations in N-terminal phosphorylation sites of β -cat (21,45-47). Selected other cancers have also been reported to have β -cat mutations (22-27).

The studies presented here were undertaken with the intent of further defining the role of APC/ β -cat/Tcf and E-cad/cad pathway defects in gastrointestinal tract cancers. Our findings indicate a role for Tcf deregulation in gastric and pancreatic cancers, resulting from mutations in β - and γ -cat in some cases, and in others from yet to be defined defects. Mutations in β -cat were found in one gastric and one pancreatic cancer cell line, and we confirmed that the mutant β -cat proteins activate Tcf transcription *in vitro*. Like most other previously described *CTNNB1* mutations in colorectal and other cancers, the mutations affect the presumptive GSK3 β phosphorylation sites of β -cat. Our studies also identified a missense mutation of a potential GSK3 β phosphorylation site in the N-terminal region of γ -cat, and we found that this mutant protein had clearly detectable but more modest effects on Tcf transcriptional activation than comparable mutant β -cat proteins. To the best of our knowledge, our studies are the first to identify activating mutations in the *CTNNG1* gene in cancer, and the findings imply that missense mutations and small in-frame deletions of the N-terminal phosphorylation sites of γ -cat are likely to have effects in cancer cells analogous to those of β -cat mutations. We failed to find *CTNNB1* or *CTNNG1* mutations in studies of a panel of 36 primary gastric cancers. Similar to colorectal cancer, where β -cat mutations are only found in about 2-5% of primary tumors (45-47), mutations in β - and γ -cat appear to be rare in primary gastric cancers. Nevertheless, our data imply that Tcf transcriptional deregulation may be important in the pathogenesis of a subset of pancreatic and gastric cancers. In some cases, such as the ASPC line, it may result from defects, other than those in the *APC*, *CTNNB1*, or *CTNNG1* genes.

We had hoped to reconcile the large body of data demonstrating frequent inactivation of E-cad by mutational or epigenetic mechanisms in many different cancer types with the recent data implicating β -cat deregulation and Tcf transcriptional activation in colorectal and other cancers. E-

cad is a major binding protein for β -cat in cells and β -cat has an important role in E-cad's cell adhesion function, via its role in linking E-cad to α -cat and the cytoskeleton. As such, we initially predicted that loss of E-cad might lead to disruption of β -cat regulation, with a resultant increase in free cytosolic β -cat and subsequent Tcf deregulation. However, this prediction was not borne out by our studies. Loss of E-cad expression in gastric, pancreatic, and breast cancers did not result in clearcut increases in β -cat levels, and no evidence of CTTA was found in cancer lines lacking endogenous E-cad expression. A possible explanation for these observations is that the proteins and pathways responsible for regulating β -cat cytosolic levels, such as GSK3 β , APC, and axin, remain fully functional in cancer cells with E-cad defects. Moreover, our findings imply that there might be no direct connection between defects in the E-cad/cat pathways and the APC/cat/Tcf pathways in cancer cells. As such, the functional consequences of inactivating mutations in APC or activating mutations in β - or γ -cat are likely to be distinct from those attributable to E-cad inactivation. Future studies will provide novel and definitive insights into the means by which β -cat functions in both E-cad cell-cell adhesion and Tcf transcriptional activation, as well as the specific means by which E-cad inactivation contributes to altered cell growth control and cancer development and progression. Perhaps then the apparently differing consequences of catenin and E-cad defects on the cancer cell phenotype will be reconciled.

ACKNOWLEDGEMENTS

The authors thank Drs. B. Vogelstein and K.W. Kinzler for generously providing the wildtype APC expression vector and the Topflash and Fopflash reporter constructs, and Drs. K.R. Cho and M. Hahn for comments on the manuscript.

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Table 1. Oligonucleotide primers for cloning and mutational analysis of β -cat and γ -cat.

Oligo Name	Sequence
BCATFL-S	5'-GAGCGAGGGGAGGCGGAGACG-3'
BCATFL-A	5'-GCCAATCACAATGCAAGTTCAGAC-3'
PLAKFL-S	5'-CCCGGTCAGGCCCCATACTCAG-3'
PLAKFL-A	5'-CTCCCCATCCCCACCAAAGACACA-3'
BCATDN90-S	5'-AGCGTGGACAATGGCTCGAGCTCAGAGGGTACGAG-3'
BCATDEX3-S	5'-AGCGTGGACAATGGCTACTCAAGATATTGATGGACAGTATGCAATGAC-3'
BCATEX2-S	5'-CGTGGACAATGGCTACTCAA-3'
BCATEX4A	5'-TGCATACTGTCCATCAATA-3'
BCATGS-S	5'-TGGGTCATATCACATTCTTTTT-3'
BCATGS-A	5'-CTCTTACCAGCTACTTGTTCCTTGA-3'
PLAKG-S	5'-GGAGCAGCCTATCAAGGTGACTGAGTGG-3'
PLAKG-A	5'-GAGACCCCCTACAATCTGCCTCCTTTCA-3'

FIGURE LEGENDS

Figure 1. Tcf transcriptional activity is deregulated in a subset of gastric and pancreatic cancer cell lines. Each of the 14 cell lines was co-transfected with 1 ug of either the pTopflash or pFopflash constructs and 1 ug of a control LacZ construct. The pTopflash construct contains 3 copies of a consensus Tcf binding site cloned upstream of a minimal promoter element and luciferase reporter gene, and pFopflash contains 3 copies of a mutated Tcf binding site cloned upstream of luciferase. Comparison of the luciferase activity of the pTopflash construct and that of the control pFopflash construct proves a measure of the relative Tcf activity. The mean and standard deviation of the luciferase activities was determined following 3 or more transfection experiments. Constitutive Tcf transcriptional activity (CTTA) is seen in the colorectal cancer lines SW480 (APC mutation) and HCT116 (β -cat mutation), as well as 2 gastric and 2 pancreatic cancer cell lines (indicated by arrows). Note that the Tcf activities for the 4 lines with very elevated activities are not shown to scale.

Figure 2. β - and γ -cat mutations detected in gastric and pancreatic cancer cell lines. A schematic representation of the 781 amino acid β -cat protein product is shown, and the sequence of the presumptive GSK3 β phosphorylation sites at the N-terminus is indicated. The β -cat mutations identified in the AGS gastric cancer and HS 766T pancreatic cancer lines are indicated. The γ -cat protein is closely related to β -cat, and its corresponding N-terminal sequence is indicated. The γ -cat mutation in the NCI-N87 gastric cancer line is noted.

Figure 3. Overexpression of APC does not suppress Tcf transcriptional activity in the ASPC pancreatic cancer cell line. The mean and standard deviations of the Tcf activities are shown. Transfections of the 3 cell lines were carried out in triplicate, essentially as described in the legend to Figure 1, except that the two different amounts of an expression construct encoding wildtype APC or a control vector were added in the transfections. Equal masses of DNA were used in each

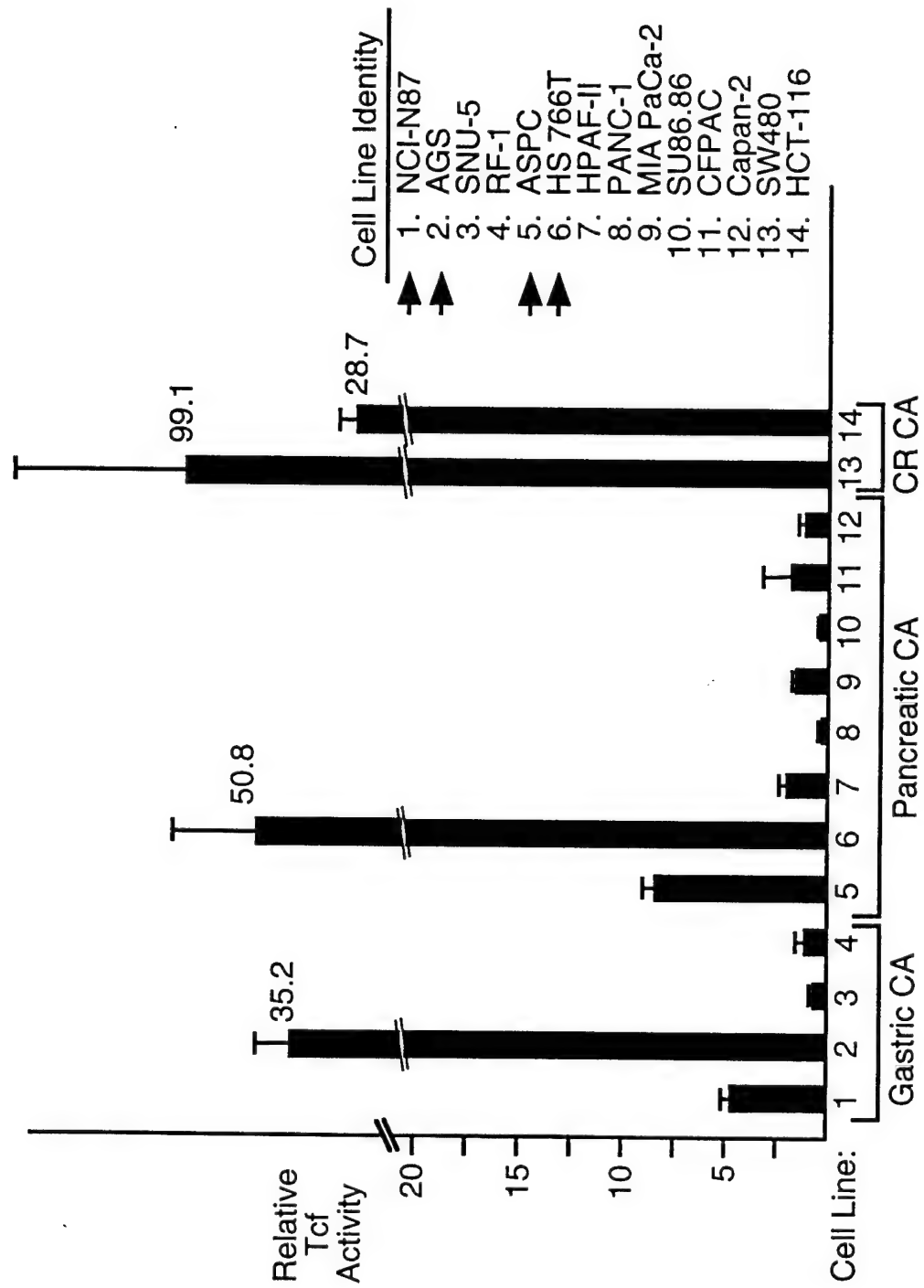
transfection. Overexpression of APC reduced the relative Tcf transcriptional activity in the SW480 colorectal cancer cells, but not in the HCT116 or ASPC cells.

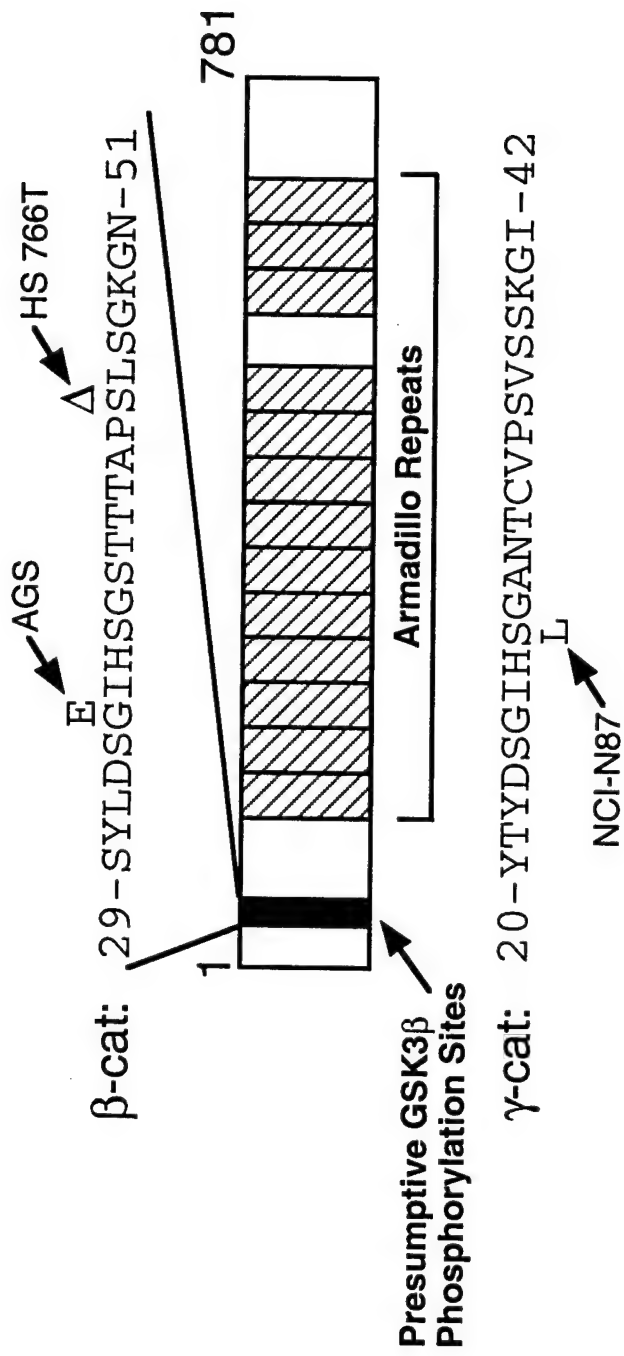
Figure 4. β - and γ -cat proteins with missense mutations and single amino acid deletions strongly activate Tcf transcriptional activity, while wildtype β - and γ -cat proteins and β -cat proteins with large N-terminal deletions are considerably less potent in activating Tcf transcription.

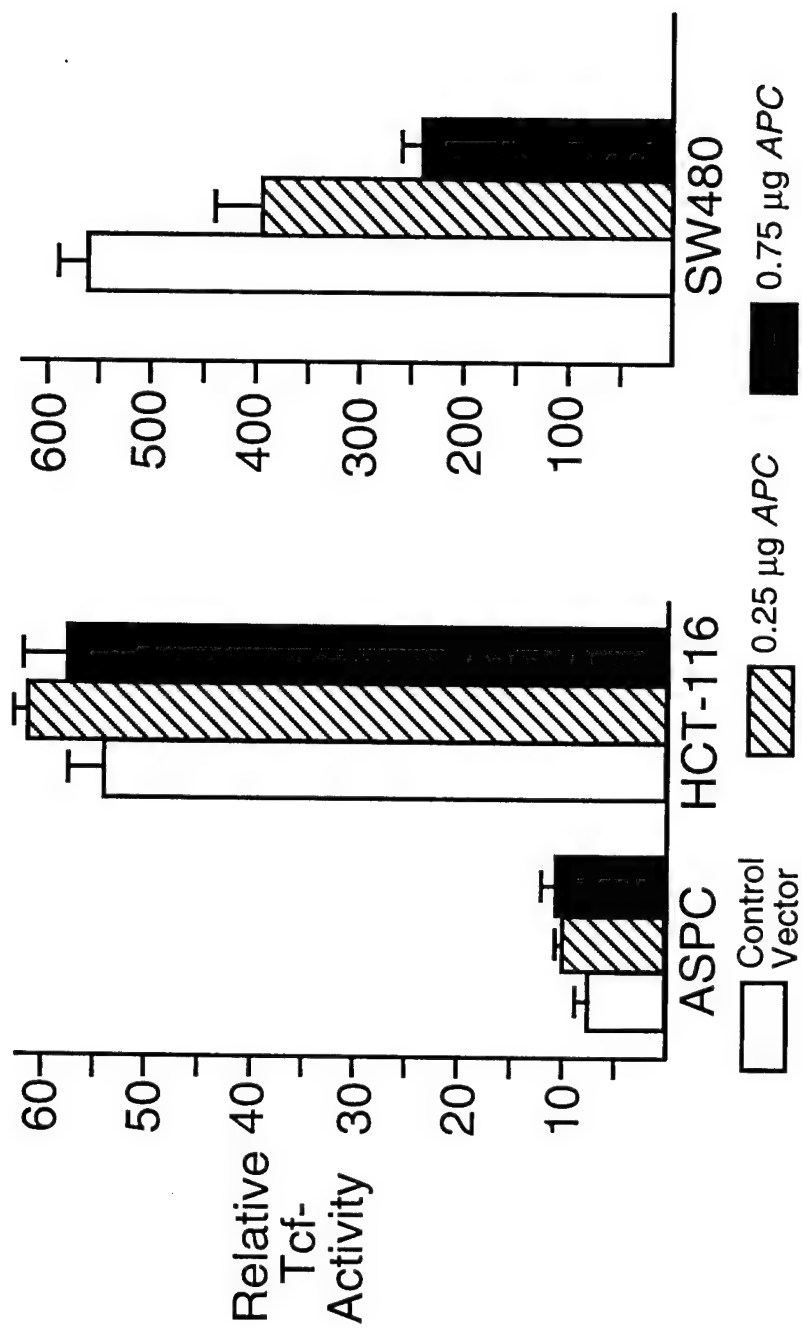
Transfections were carried out in 293 kidney cells as described in the legend to Figure 1, and the mean and standard deviation of Tcf activities from triplicate experiments are indicated for the respective constructs. Note that the Tcf activity of the S33Y construct is not shown to scale.

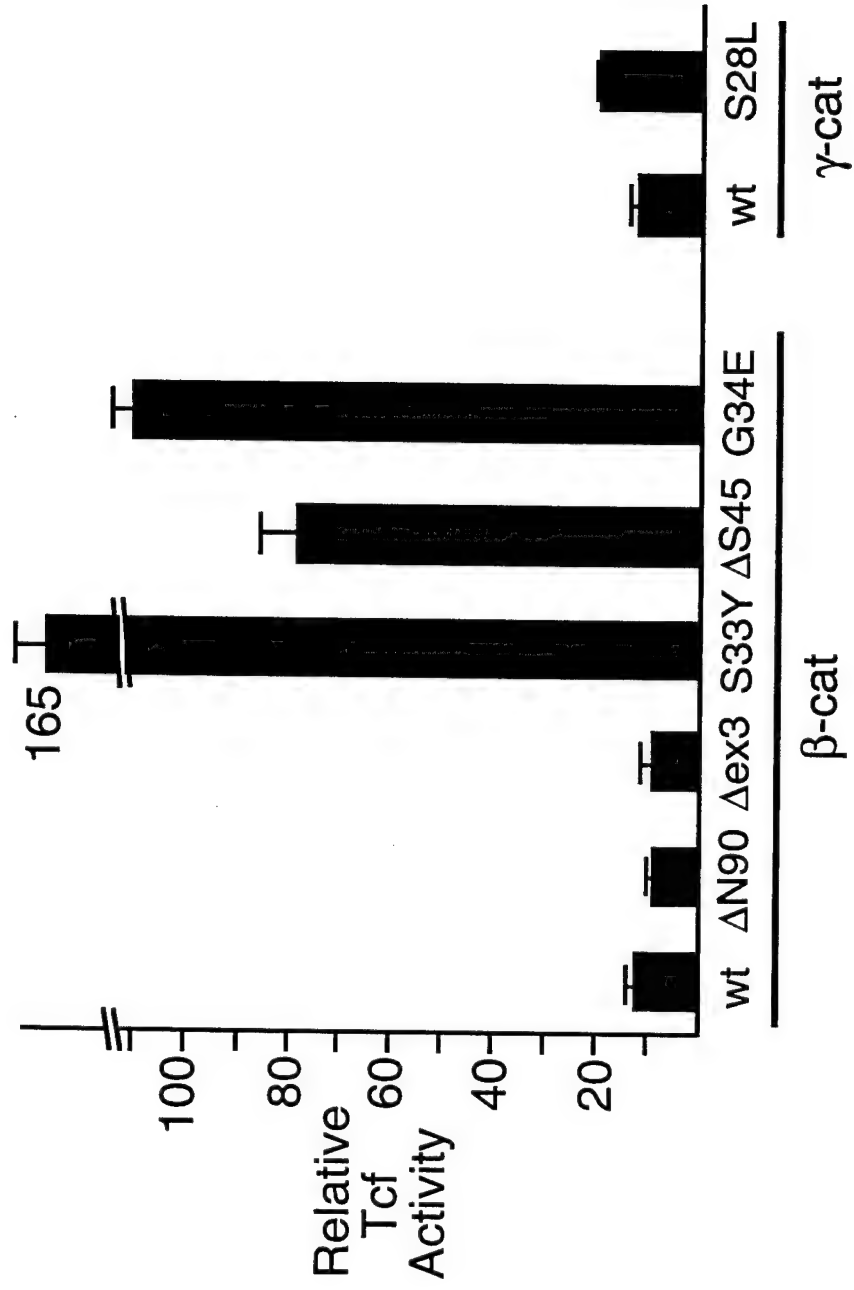
Figure 5. ECL-Western blot studies reveal alterations in the expression of E-cad and catenins in selected gastric and pancreatic cancer cell lines. Protein lysates were prepared from the cell lines growing at roughly 70-85% confluence. The lysates were subjected to SDS-PAGE and multiple immunoblots were generated in parallel. ECL-Western blot analysis was carried out using antibodies against E-cad, α -cat, β -cat, γ -cat, and GSK3 β , as well as a control protein - Na⁺/K⁺-ATPase.

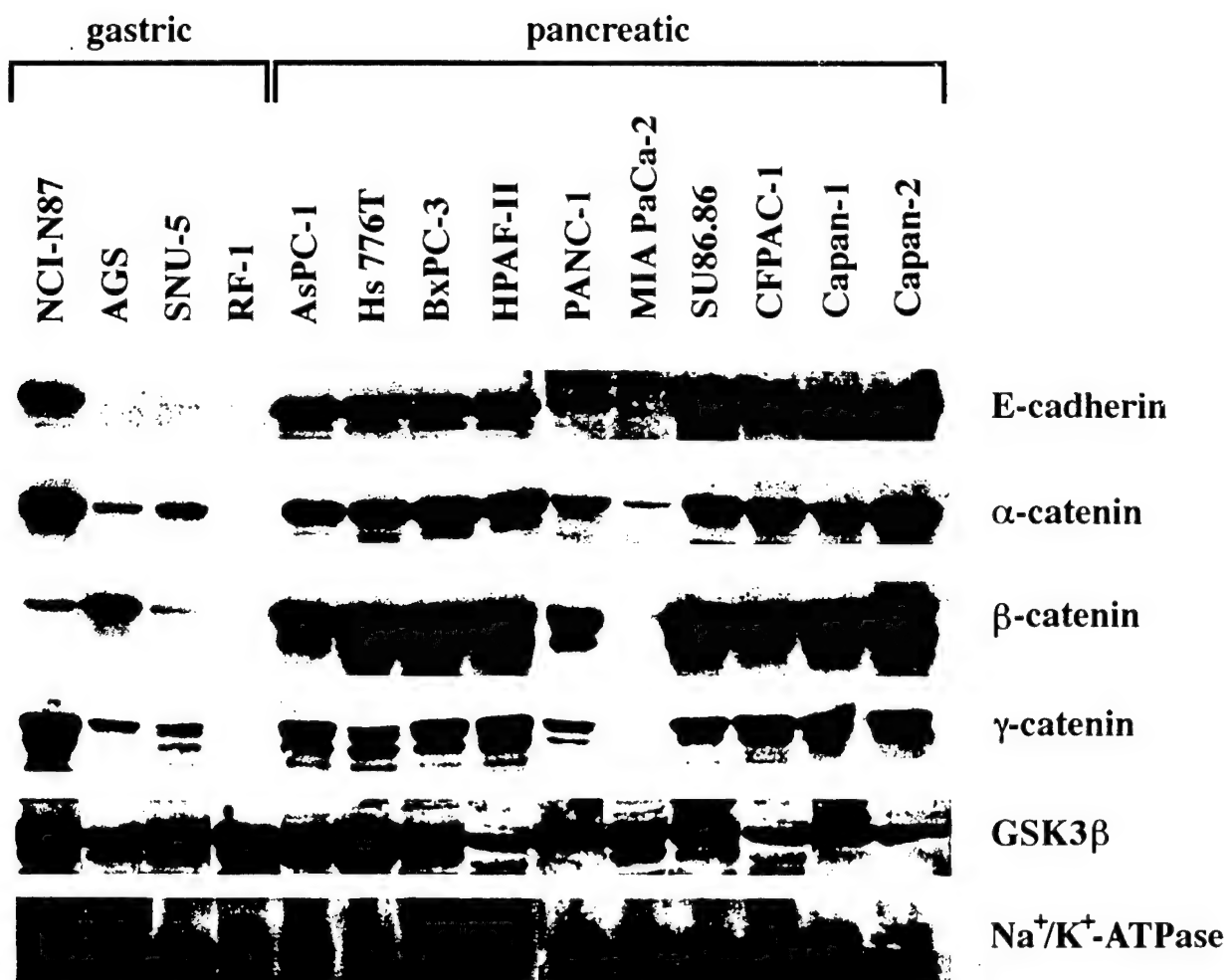
Figure 6. Mutant β - and γ -cat proteins potently activate Tcf transcriptional activity in the BT549 breast cancer cell line. Transfections were carried out as described in the legend to Figure 1, and the mean and standard deviation of Tcf activities from triplicate experiments are indicated for the respective constructs.

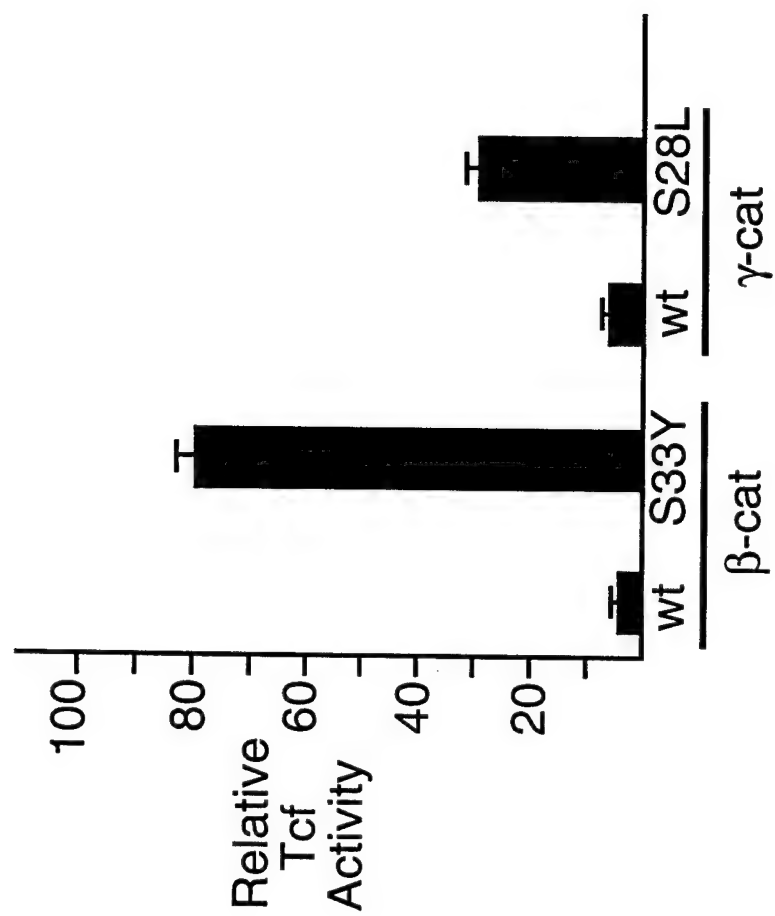












Met Expression is Associated with Poor Outcome in Node-Negative Breast Cancer

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Grant Support: This work is supported by grants from the William and Catherine Weldon
Donaghue Foundation for Medical Research, NIH RO-1 GM57604 and the US Army
(DAMD17-94-J4366)

pages: 15

tables: 3

figures: 3

This work will be presented at the United States and Canadian Academy of Pathology Meetings
in San Francisco on March 23, 1999.

Precis:

Expression of *Met*, the receptor for hepatocyte growth factor/scatter factor as been associated with aggressive behavior in a wide range of cancers. This paper shows that it is an independent predictor of poor outcome in node-negative breast cancer patients.

Abstract:

Background: Activation of the receptor for scatter factor / hepatocyte growth factor, *Met*, is associated with mitogenesis, motogenesis, and decreased cell adhesion. Elevated expression of *Met* has been shown in advanced cases of prostate, gastric, pancreatic, and thyroid cancer. We have previously demonstrated that *Met* expression is an independent prognostic marker associated with decreased survival in breast carcinoma.

Methods: Expression of *Met* in 113 archival breast cancer specimens from patients with node-negative invasive ductal carcinoma was evaluated using standard immunoperoxidase technique. Cases were scored by two pathologists using an H-score algorithm and then analyzed for correlation with known prognostic factors and survival.

Results: Expression of *Met* showed a bi-modal distribution with 25% of the cases showing high levels of expression. In contrast to previous studies, we find a significant association between *Met* expression and nuclear and histologic grade. The five year survival for *Met* negative patients is 95% in this cohort, compared to 80% for *Met* positive cases. Patients whose tumors have a high level of *Met* expression have a five-year relative risk of dying of metastatic disease of 5.05 ($p = 0.03$), independent of patient age and tumor size. Combined analysis of *Met* and nuclear grade results in a marked increase in independent predictive value ($RR = 33.4$, $p < 0.01$).

Conclusions: We find that high levels of *Met* expression are associated with death due to metastatic disease in lymph node-negative breast carcinoma. Expression of *Met* may be a useful prognostic indicator of more aggressive disease in patients whose prognosis is not easily stratified by current histopathologic markers.

Key Words: breast cancer, immunohistochemistry, hepatocyte growth factor/scatter factor receptor, prognosis, Metastasis, *c-met*,

Introduction:

As many as 20% of women with lymph node negative breast carcinoma will ultimately die of metastatic disease ¹. Many studies have sought to identify such patients using a variety of prognostic factors, with the ultimate goal of reserving the most aggressive therapy for patients at greatest risk for disease progression. Initially, histologic parameters, including tumor size, histologic and nuclear grade, ploidy, and tumor necrosis were analyzed for their prognostic benefit, and although associations have been found ^{1 2 3}, none of these methods has been a consistently powerful predictor of outcome. Subsequently, estrogen and progesterone receptor expression were used both as a prognostic indicators and as markers for the potential response to hormone therapy (i.e. tamoxifen) ^{4 5}. Recent studies have sought to define mutations and/or amplifications of tumor-related genes. In particular, the amplification of HER2 (neu, erbB-2) -- a homologue and dimeric partner of epidermal growth factor receptor -- is associated with aggressive disease as well as resistance to chemotherapy ^{6 7}. The identification of such tumor-related genes provides the opportunity to develop specific treatment strategies based on the phenotype of individual patient's tumors. In the case of HER2, anti- HER2 immunotherapy has shown initial promise in combating breast cancer ⁸.

Several studies have shown that *Met* is also over-expressed in aggressive forms of other tumors including prostate, thyroid and gastric carcinomas ^{9 10 11 12 13}. *Met* is a dimeric tyrosine kinase growth factor receptor ^{14, 15}, which is primarily found on epithelial cells ¹⁶. Its ligand, HGF/SF is a mesenchymal-derived cytokine which induces epithelial cell dissociation, motility, and mitogenesis in a paracrine fashion ¹⁷. In the breast, *Met* is expressed in normal ductal epithelium and functions in both the embryonic development and subsequent functional remodeling of the breast ^{18 19 20}.

Like *Met*, high level expression of HGF/SF is found in aggressive breast cancers ^{20, 21} and is associated with survival ²². Some studies have shown that the HGF/SF in such tumors is derived from reactive stroma rather than the carcinoma itself, suggesting cross-talk between tumors and host-derived mesenchyme ²³. Alternatively, some groups have found HGF/SF

expression in neoplastic epithelium (as well as benign)^{24 25 26}, indicating that *Met*-HGF/SF can also form an autocrine loop which drives tumor growth. The underlying reason for the up-regulation of *Met* in tumors may be explained by mutations of the *Met* gene (as seen in papillary renal cell carcinoma,²⁷) or by variations in *Met* RNA splicing²⁸ or by other, still unknown mechanisms.

In our pilot study with 45 node-negative patients²⁹ it appeared that the expression of *Met*, as gauged by immunofluorescence, would have prognostic value even in node-negative patients. To validate the predictive value of *Met* expression in patients with lymph-node-negative breast carcinoma, we analyzed tumors from a cohort of 113 patients, using standard immunoperoxidase technique. We demonstrate that *Met* has significant predictive value in determining the outcome of these patients. In contrast to our previous studies as well as those of others, we also show that the expression of *Met* is associated with nuclear grade.

Materials and Methods:

Polyclonal Antibodies

The anti-*Met* polyclonal antibody, #sc-10, (Santa Cruz Biotechnology Inc., Santa Cruz, CA) is affinity-purified rabbit IgG raised against a synthetic peptide corresponding to the last 12 amino acids at the carboxy-terminal of the human *Met* beta subunit.

Patient Population

The cohort consists of 113 patients who underwent breast resections at Yale-New Haven Hospital for invasive ductal breast carcinoma from 1983 to 1993. All had axillary lymph node dissections without evidence of lymph node metastasis. The age at diagnosis ranged from 32-84 yr (mean of 50.9 yr). Patients were followed for a maximum of 60 mo. with a mean follow up time of 50.9 mo. 62.5% of the patients were followed for the full 60 mo., and the overall survival was 90.2%, which is consistent with published survival statistics^{30 1}. Clinicopathologic

parameters collected on each case include age, tumor size, tumor histologic and nuclear grade, ER status, ploidy, and survival. To complete the analysis, nuclear grade and histologic grade on some cases were assigned retrospectively by a pathologist (R.L.C) in a blinded manner, and according to the methods described by Fisher et al.³¹. All cases collected for this study are new cases, specifically this cohort does not include the 45 node-negative patients from our previous study²⁹. All material was collected under the auspices of the Critical Technologies Program at Yale and in accordance with human investigation committee protocol 8219 to the principal investigator (D.L.R.).

Immunostaining

Sections were obtained from archival formalin-fixed, paraffin-embedded tissue blocks from each case. Sections were baked at 60°C overnight, deparaffinized with xylene, rehydrated, and then blocked with a mixture of methanol and 30% hydrogen peroxide and prepared for immunostaining using a pressure cooker antigen retrieval method³². Slides were incubated for 1 hr at room temperature in a humidity tray with a 1:250 dilution of the polyclonal *Met* primary antibody (Santa Cruz Biotechnology, Inc.) in 0.3% bovine serum albumin in tris-buffered saline (TBS). After incubation slides were washed 7 times with TBS including 0.01% triton X-100 in the sixth wash. Slides were processed using an anti-rabbit Vector ABC kit (PK4001), and developed with diaminobenzidine for 10 min at room temperature (Vector Laboratories, Burlingame CA).

Histologic Scoring and Analysis

Cases were examined using standard microscopic technique by two pathologists (R.L.C. and D.L.R.). In each case, a serial hematoxylin and eosin stained section from the same block was examined for orientation and confirmation of the histologic diagnosis. Each case was scored blindly with respect to patient history, presentation, and previous scoring. Staining for *Met* protein was evaluated in the tumor as well as in the normal breast tissue, when present. Examples of a

high expressing case and a low expressing case with adjacent positive control expression in normal duct are shown in figure 1. Each tumor sample was scored by the cross-product (H-score) of the percentage of tumor cells staining at each of three staining intensities. For example, a particular tumor may have 30% of the cells staining at one-plus and 70% of the cells staining at three-plus, for a combined H-score of $[(30 \times 1) + (70 \times 3)] = 240$ out of maximum of 300. There was good correlation in the scores given by both pathologists (81% of the cases showed agreement within a 40 point range). Cases where there was greater than a 50 point scoring discrepancy were rescored and reconciled on a two headed microscope. Final H-scores on each case represent the average of scores by each pathologist.

Statistical analysis

Patient follow-up information was obtained the Yale Comprehensive Cancer Center and the Connecticut Tumor Registry. The association of various disease parameters with *Met* expression was analyzed using standard statistical techniques using Statview 4.5 software (Abacus Concepts, San Francisco, CA).

Results:

Scores of the expression of *Met* in invasive ductal carcinoma in this cohort showed a bimodal distribution clearly dividing the cohort into high and low expressers (figure 2). Twenty-five percent of the cases (28) had scores greater than 180 and were designated as "*Met* positive" while the remaining cases (85) were considered "*Met* negative" for analysis.

We used the Pearson chi-square test to determine if *Met* expression was related to other histopathologic indicators of aggressive disease. *Met* expression was strongly associated with both high nuclear and histologic grade, but not with tumor size, patient age, estrogen receptor status, or ploidy (table 1). As estrogen receptor status and ploidy analysis were performed on only one-half of the cases, these parameters were excluded from multivariate analysis.

The relationship between *Met* expression in tumors and survival was assessed using the Kaplan-Meier method. Significantly decreased survival was observed in patients with high *Met* expressing tumors; the 5 year survival was 80% compared to 96% in the *Met* negative group (log rank $p = 0.012$) (Figure 3).

To determine the independent predictive value of high *Met* expression and compare it with some established prognostic factors, we used the multivariate Cox proportional hazards model. As nuclear and histologic were strongly associated with *Met*-positivity, these criteria were initially excluded from the analysis. Women with a positive *Met* score had a relative risk of 5.05 for breast cancer mortality compared to women with *Met*-negative tumors (95% C.I. 1.19-21.3) (table 2). High nuclear grade (III/III) was the only traditional histopathologic parameter which showed independent prognostic value, with a relative risk of 7.54 (95% C.I. 1.59-35.8). Previous studies of nuclear grade in node negative breast carcinoma have found lower relative risks 2.6 to 3.2^{2 33}; however, these were within the 95% confidence interval of this study. Because of the strong association between *Met*-expression and nuclear grade we had limited power to assess the independent association of each prognostic factor with risk of breast cancer mortality. As an alternate method of analysis, we determined the increased risk of mortality among women whose tumors were both *Met*-positive and had a high nuclear grade, compared to women with *Met*-negative tumors of low (I-II/III) nuclear grade. This analysis revealed a marked increase in the relative risk, which was independent of histologic grade, age, and size (33.4; 95% C.I. 3.27-340) (table 2). Five year survival for this group (*Met* positive, nuclear grade 3) is 62%, compared to 97% for *Met* negative, nuclear grade 1 or 2 ($p < 0.001$).

Discussion:

Our studies indicate that the expression of *Met* in invasive breast carcinoma is of significant predictive value in determining patient survival. Similar to our earlier study on *Met* expression in a combined cohort of node-negative and node-positive patients²⁹, we found a statistically significant association between high levels of *Met* expression and poor outcome. Furthermore, our

results suggest that the predictive value of *Met* positivity is independent of tumor size and patient age. In contrast to that study, in this node-negative cohort we find a relationship between *Met* expression and high nuclear and histologic grade. Other studies of *Met* in breast carcinoma have looked at histologic grade in combined cohorts of node-positive and node-negative patients and found no association^{20, 34}. Although we found a strong association between *Met* expression and nuclear grade, there was not complete concordance. Thus, combining nuclear grade and *Met* score resulted in a marked increase in the independent predictive value of nuclear grade alone, demonstrating that the evaluation of *Met* expression augments the standard histopathologic markers.

The association between *Met* and high nuclear and histologic grade is similar to that seen with HER2³⁵. Such an association is perhaps not surprising given that HER2 and *Met* induce proliferation, and that mitotic activity is closely associated with high nuclear grade^{33 36}. Although examination of HER2 was beyond the scope of this study, we are currently investigating a possible interaction between these two markers.

Even though we had follow-up in excess of 10 years on a small percentage of cases, we chose to focus our study on five year survival. We have found that some patients that were *Met* negative at diagnosis showed high levels of *Met* expression in late recurrences. In this cohort, we were able to analyze the *Met* expression of six recurrent and/or metastatic lesions in patients whose tumors were originally *Met* negative. In all six cases, the recurrence/metastasis expressed markedly higher levels of *Met* (above 180 in five of the six). This finding is similar to that from our previous study with a completely non-overlapping cohort²⁹. Although this data is insufficient to conduct statistical tests, it suggests that *Met* expression is associated with tumor aggressiveness, and raises the possibility that late recurrences in breast cancer may represent selection of high *Met* expressing clones. We are presently studying this possibility. Given the infrequent deaths from metastatic disease in patient with node-negative carcinoma (a total of 8 in this study), larger studies with long term follow up will be needed to firmly establish the value of *Met* expression at later time points.

Since *Met* is expressed at high levels in normal breast epithelium, it would seem paradoxical that high levels of expression in carcinoma confers a worse prognosis. This is particularly true given that in vitro studies have shown that *Met*-HGF/SF signaling induces morphogenesis of tissue-specific, organized structures (e.g. tubules, crypts, and alveoli) ³⁷. On a more basic level however, the construction of such organized structures would involve individual cellular and molecular processes which are also associated with tumor aggression, namely proliferation, protease production, and stromal "invasion" of epithelial cells, as well as the development of feeder blood vessels.

Admittedly, looking for over-expression of *Met* on tumor cells is only an initial step in understanding the complex interplay of *Met* and HGF/SF in promoting carcinogenesis. This is particularly true given the intriguing hypothesis that some tumors develop from *Met* expressing normal epithelium, grow initially as *Met*-deficient lesions, and subsequently progress as aggressive, *Met*-expressing tumors. Indeed recent studies have begun to define the events associated with the gain or loss of *Met* function and/or expression. Lin et al. have shown that *Met* down-regulation can occur by an alternative splice which deletes the AUG-containing exon 2 ²⁸. Such an event may occur early in the development of carcinoma and make tumors insensitive to the regulation of HGF/SF-induced differentiation. Subsequent mutations may induce the re-expression and constitutive activation of *Met*, resulting in enhanced tumor aggressiveness. In support of this later event, studies of papillary renal cell carcinoma have found point mutations in *Met* which may alter its kinase function ²⁷. We are currently looking for such mutations in cases of breast carcinoma. Regardless of the mechanisms, however, the strong correlation between *Met* expression and survival described here clearly support the hypothesis that the *Met*-HGF/SF pathway plays an important role in the progression of breast carcinoma, and that high levels of *Met* expression are prognostic of disease progression and death among women with node-negative tumors.

Acknowledgments:

The authors thank Thomas D'Aquila for his expert technical assistance, and Dr. Diane Fisher for providing clinical information from the Connecticut Tumor Registry.

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Figures:

Figure 1 *Met* expression patterns in examples of high and low expressing cases of invasive ductal carcinoma. Tumors were stained with polyclonal antibody to *Met* and developed with standard immunoperoxidase/diaminobenzidine technique. A) shows an example of a case with an H score of 300 with adjacent stromal tissue that does not stain. B) shows an example of a case with invasive tumor on the left with a low H-score with adjacent strongly staining normal ducts as an internal positive control. Scale bar = 50µm.

Figure 2 H-scores for *Met* staining on tumors appear in a bimodal distribution. Staining was recorded using an H-score analysis (see materials and methods), with a range of 0 to 300 points. There was a clear bimodal distribution, and tumors with scores below 180 were designated as *Met*-negative, and those above 180 as *Met*-positive.

Figure 3 Kaplan-Meier survival analysis demonstrates that *Met* has significant predictive value for breast carcinoma survival. The five year survival for patients with *Met*-negative tumors was 96%, compared to 80% for *Met* positive ones. This difference is statistically significant ($p = 0.012$) as determined with the Mantel-Cox log rank test.

Table 1: Chi-Square Analysis

Histopathologic Criteria	Met Positive (n=28)	Met Negative (n=85)	Total Pop. (n=113)	P Value
High nuclear grade (III/III)	12 (43)	10 (13)	22 (21)	<0.01
High histologic grade (III/III)	11 (39)	14 (18)	25 (23)	0.02
Estrogen receptor negative*	7 (64)	15 (34)	22 (40)	0.07
Aneuploid*	7 (78)	17 (52)	24 (57)	0.16
Age < 50 yr.	7 (26)	15 (18)	22 (20)	0.36
Size > 2 cm	17 (63)	45 (54)	62 (56)	0.43
Dead of disease	5 (18)	3 (4)	8 (7.1)	0.01

The dotted line separates statistically-significant from non-significant associations

*Analysis performed on approximately half of the specimens

Population percentages are displayed in parentheses

Table 2: Multivariate Analysis

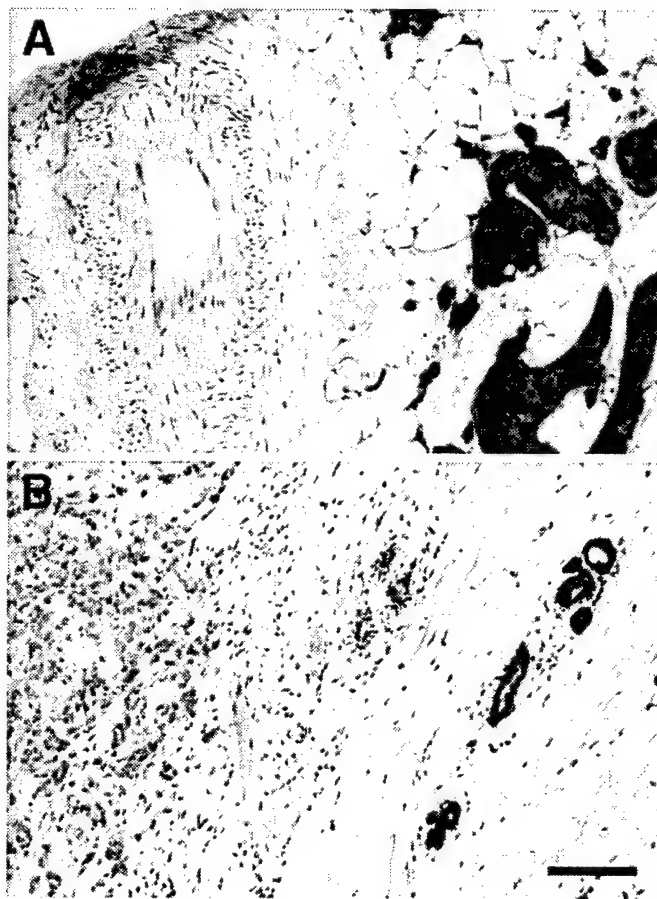
Variable	Relative Risk	95% Confidence Interval	P Value
<i>Met</i> positive	5.05	1.19-21.3	0.03
Age < 50 yr.	2.43	0.56-3.82	0.23
Size > 2 cm	1.11	0.26-4.72	0.88
High nuclear grade	7.54	1.59-35.8	0.01
Age < 50 yr.	2.61	0.61-11.2	0.20
Size > 2 cm	1.14	0.27-4.81	0.86
High histologic grade	0.84	0.18-3.93	0.82
<i>Met</i> pos / HNG	33.4	3.27-340	<0.01
Size > 2 cm	3.86	0.56-26.7	0.17
Age < 50 yr.	2.24	0.27-18.9	0.46
High histologic grade	0.32	0.03-3.53	0.35

Met pos / HNG: *Met*-positive tumors with nuclear grade III/III compared to *Met*-negative tumors with nuclear grades of I-II/III.

Table 3: *Met* Scores of Subsequent Recurrent or Metastatic Lesions

Patient Number	Primary <i>Met</i> score	Site of recurrence / Metastasis	Time interval (months)	Recurrence / Metastasis <i>Met</i> score	Dead of disease
7	0	Ipsilateral axillary LN	48	280	No
11	280	Lung	4	250	Yes
18	133	Chest wall (multiple)	10/18/31	50/200/280	Yes
24	43	Contralateral breast (no intraductal component)	88	300*	No
51	33	Chest wall	60	200	No
110	28	Skull	56	250	No
131	0	Chest wall	7/8	150	Yes

* only a few glands were present for evaluation



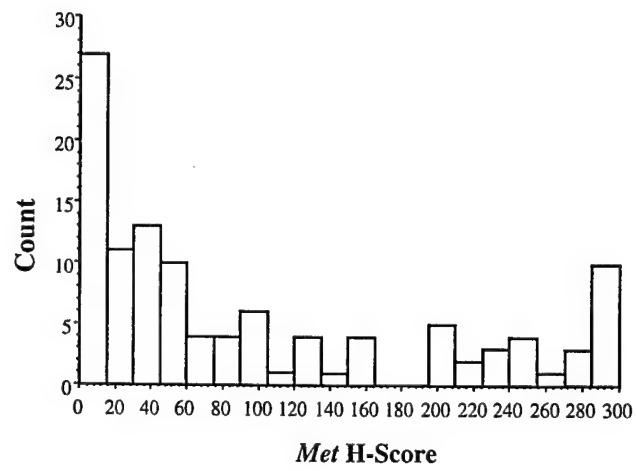


Figure 2
Camp et al. 1999

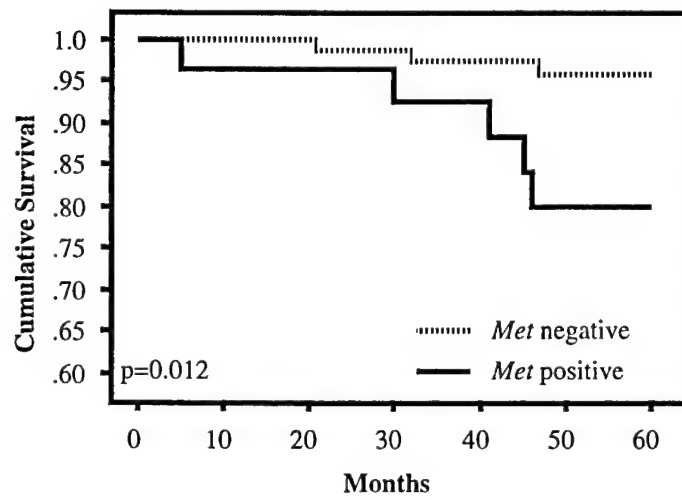


Figure 3
Camp et al. 1999

E-cadherin expression is a sensitive and specific method for detection of carcinoma cells in fluid specimens

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Key Words: adhesion, cadherin, effusion, cytology, cytopathology, Thin Prep™

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Grant Support: This work is supported by grants from the William and Catherine Weldon Donaghue Foundation for Medical Research, NIH RO-1 GM57604 and the US Army (DAMD17-94-J4366)

This work was presented as a poster at the American Society of Cytopathology Meeting in Nashville in November, 1998

pages: 11

tables: 3

figures: 1

Précis

E-cadherin expression is a highly sensitive and specific method for detection of carcinoma cells in fluids. Although the sensitivity of E-cadherin alone exceeds that of morphologic cytology alone, the combination with morphology results in an assay with exceptionally high sensitivity and specificity.

Abstract:

Introduction: The distinction between reactive mesothelial cells and carcinoma in pleural, peritoneal and pericardial fluids is often difficult. We have previously shown that E-cadherin, an epithelial specific adhesion protein, can be useful for this distinction. In this study we test the sensitivity and specificity of E-cadherin compared to conventional cytology for assessment of carcinoma in fluids

Methods: After routine diagnosis, a second Cytoc Thin Prep™ slide was prepared on a series of 102 sequential fluids received by our service during 2 time periods. E-cadherin expression was assessed using routine immunologic techniques.

Results: Of 102 cases assayed, 71 showed no evidence of E-cadherin staining and 31 showed unequivocal positive staining. Sensitivity and specificity was determined for both E-cadherin and cytomorphology independently and together. If both cytomorphology and E-cadherin were used together, a definitive and correct diagnosis could be made on nearly every case in this study (sensitivity 92%, specificity 100%).

Conclusions: The E-cadherin expression assay has sensitivity and specificity comparable to conventional morphologic diagnoses. When used as an adjunctive test with cytomorphology, the resultant sensitivity and specificity approach 100%.

Introduction:

The keystone protein in the establishment and maintenance of epithelial cell adhesion and tissue integrity is E-cadherin. It is a 120 kD transmembrane glycoprotein whose calcium sensitive homotypic adhesion is the primary stabilizing event in cell-cell adhesion¹. It also serves as a signal for polarization or other cellular differentiation events². It is a member of the broad cadherin family of adhesion proteins which includes greater than 20 different cadherins from a range of tissues. Recently it has been cited in the pathology literature in two different roles. 1) Decreased levels of expression or loss of expression of the protein has been described in many tumors and in some, decreased expression levels correlate with outcome³. 2) Cadherin expression has been used in diagnostic pathology to differentiate the origin of morphologically similar lesions. Specifically, Han and colleagues have used E- and N-cadherin to distinguish malignant mesothelioma from adenocarcinoma⁴.

A related diagnostic problem in cytopathology is the distinction between benign reactive mesothelial cells and well differentiated carcinoma in pleural, peritoneal and pericardial fluids. Since E-cadherin is expressed only in cells of epithelial lineage, it can be very useful in making this distinction. Previous studies^{5, 6} including one study by the Abati group (abstract presented at USCAP meeting, Mar. 1998) have shown that anti-E-cadherin antibodies are valuable to distinguish epithelial malignancies from reactive mesothelial cells. The purpose of this study was to determine the sensitivity and specificity of the E-cadherin antibody to distinguish malignancy, independently and in combination with morphologic cytopathology.

Methods:

One hundred and two specimens were randomly selected from two different time periods between 6/2/97 to 9/10/97 and 2/6/98 to 5/7/98. The cases included 48 pleural fluids, 50 peritoneal fluids and 4 pericardial fluids. All pleural, pericardial, and peritoneal fluids were submitted for routine cytologic examination in the usual manner. The specimen was spun down and the supernatant was discarded. The pellet was then resuspended in CytoLyt™ (Cytoc Corp. Boxborough, MA) and re-centrifuged. The supernatant was then discarded and 2 drops of the pellet were added to PreservCyt™ solution. After 15 minutes, the specimen was processed using the Cytoc Thin Prep Processor™. Finally, the specimens were stained using standard Pap staining technique for routine screening by the cytotechnologist and sign-out by the pathologist.

In order to evaluate E-cadherin expression, at least 2 additional slides were made from each case. One was treated with E-cadherin antibody and the other was used as a negative control. Thin-Prep slides were rinsed for 5 minutes in tap water followed by 5 minutes in Tris buffered saline(TBS) (150 mM NaCl, 20mM Tris pH=8). Slides were then blocked for 20 minutes with diluted normal serum from Vectastain ABC-AP Kit (Vector Laboratories, Burlingame, CA). After blocking, slides were washed once in TBS. E-cadherin monoclonal antibody (Transduction labs, Lexington, KY) was diluted 1:250 in TBS, 3-4 drops applied per slide, and incubated for 30 minutes. Slides were then washed again in TBS for 5 minutes. Then 3-4 drops of diluted biotinylated antibody from the Vectastain ABC-AP kit were applied per slide and incubated for 30 minutes, followed by a 5 minute wash in TBS. Vectastain ABC-AP Reagent was added(3-4 drops) and slides were incubated for an additional 30 minutes, followed by a 5 minute wash in TBS. Vector Red Alkaline Phosphatase Substrate (Vector Laboratories) was applied(3-4 drops) and slides were incubated for 10 minutes. Slides were then washed for 5 minutes in tap water, counterstained with hematoxylin and coverslipped

The slides were viewed by a cytotechnologist and a pathologist and independently scored for E-cadherin staining as present(+) or absent(-). There were 7 discrepancies between the cytotechnologist and pathologist (7%). These were resolved viewing questionable slides on a two-heading scope and coming to a consensus opinion.

Results:

In nearly all cases of carcinoma, tumor cells showed a strong positive membrane or cytoplasmic reaction product (Figure 1). Reactive mesothelial cells showed essentially no Vector Red reaction product, similar to that seen in control specimens (without E-cadherin antibodies). Rare non-epithelial tumors showed variable staining, but were generally negative. Examples of a range of cases are shown in figure 1. Benign lesions such as endometriosis show E-cadherin expression (Figure 1C), as do a range of malignant epithelial tumors (figure 1D-H). Non-epithelial lesions including mesothelioma and lymphoma (figure 1A-B) and reactive mesothelial cells (figure 1I) show no evidence of E-cadherin expression.

E-cadherin staining was observed in 31 cases of which 29 were proven carcinomas and 2 were from patients with endometriosis. E-cadherin staining was not observed in 7 tumors which were not expected to express this protein (non-epithelial malignancies including a mesothelioma, neuroblastoma, and others). The distribution of E-cadherin expression with respect to the patients cancer status is shown in Table 1. The calculated sensitivity and specificity using only E-cadherin (without regard to morphology) to predict the presence of malignant cells in the fluid is 87.5% and 95% respectively.

The cytologic diagnosis was accurate in most cases, but included 10 cases that were called atypical. Eight of these cases proved to be positive for malignancy, while the others included one case of endometriosis and one case of reactive mesothelial cells. For purposes of calculation of sensitivity and specificity, cases signed out as atypical were

reclassified as positive if the signout included a note suggesting a high level of suspicion. The remainder of the cases (2) were re-classified as negative. Sensitivity and specificity of cytomorphologic diagnosis alone is 82% and 100% respectively, shown in Table 2. Of the 7 false negative cases, 4 were cases of carcinoma where cells were detected in the fluid by testing for E-cadherin expression, while 2 of the other 3 cases were tumors where the number of malignant cells shed into the fluid is very low (a leiomyosarcoma and a pseudomyxoma peritonei).

Given this result and the fact that immuno-stains are routinely used as ancillary studies, we calculated the sensitivity and specificity of the best final diagnosis, combining the cytomorphologic diagnosis with the information from the E-cadherin assay. The sensitivity and specificity of the combined best diagnosis is 92% and 100% respectively (Table 3).

Discussion:

A wide range of antibodies has been used in the past to attempt to make the distinction, between reactive mesothelial cells and metastatic carcinomas. The subject has been recently reviewed by Dr. Bedrossian⁷. One of the oldest immunocytochemical methods, and perhaps still the most common is the use of CEA (carcinoembryonic antigen)⁸. This antigen, in a panel with EMA, BerEP4, B72.3 and LeuM1 have been used as a panel to detect epithelial malignancies in a background of mesothelial cells⁹. Even using this or other such panels¹⁰, some tumors are still not identified, resulting in test sensitivity in the 80-85% range¹¹. Other studies where nearly all epithelial malignancies stain (such as EMA), mesothelial cells are occasionally positive as well¹². The potential advantages of E-cadherin over these panels are; 1) equal or better sensitivity may be obtained without other antigens, and 2) its unique expression patterns lead to very high specificity. While other members of the cadherin family are expressed in many cells types¹,

E-cadherin is expressed only in tissues of epithelial differentiation with no published examples of expression in mesenchymal tumors or tumor cell lines or any other non-epithelial cell or cell line.

The results from this current study with E-cadherin confirm the expression pattern in tumors of epithelial lineage. There were 3 false positive E-cadherin cases, two of which were cases of endometriosis (see figure 1C for example) and one case which is yet to be resolved, as the patient has known lung cancer but no evidence of pleural involvement to date beyond our experimental case. As seen elsewhere, E-cadherin, does not stain any non-epithelial cells, rather positives arise from non-malignant cells of epithelioid lineage in the fluid specimens. This was also seen in our previous study⁶ which included pelvic wash specimens where fragments of skin from the excision were identified by E-cadherin staining.

There were a total of 11 cases of tumors that did not show E-cadherin expression. Of those, 7 were not of epithelial lineage, and would not be expected to be detected by this assay. Three of the remaining 4 cases had very low cellularity and the tumors cells were not represented on the E-cadherin stained slide. One case showed tumor cells that were E-cadherin negative. Complete loss of expression of E-cadherin has been documented in gastric cancer¹³ and lobular carcinoma of the breast¹⁴, but is not common. More commonly, tumors show decreased E-cadherin expression, but not complete loss^{15,3}.

In order to calculate the sensitivity and specificity of this assay, we chose 102 cytology cases at random in 2 consecutive series to be representative of the normal population of cases seen by the Yale Cytopathology Service. The yearly percentage for abnormal cases seen by our service in fluids was 18.8% In this study 23.5% of the cases were positive, suggesting that this population is representative of what is typically seen in a cytology service in a tertiary care hospital. We calculated the sensitivity and specificity of E-cadherin expression for detection of malignancy, of cytomorphology for detection of malignancy, and of the combined best estimate of final diagnosis using both

cytomorphology and/or cadherin expression. The sensitivity and specificity for cadherin expression for detection of malignancy was 87.5% and 95% respectively. For cytomorphology and detection of malignancy, the sensitivity and specificity was 82% and 100% respectively. Finally, the combined best estimate of final diagnosis was 92% and 100% respectively. We conclude that E-cadherin is a valuable ancillary tool for use in distinguishing carcinoma from reactive mesothelial cells in difficult peritoneal, pericardial and pleural fluid cases.

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Figure Legends:

Figure 1. Examples illustrating cases scored as positive or negative expression of E-cadherin. Frames A, B, and I were scored as negative and the remainder of the cases are positive. The cases, by diagnosis are as follows: A) Mesothelioma, B) Lymphoma, C) Endometriosis, D) Squamous Cell Carcinoma, E) Adenoid Cystic Carcinoma, F) Adenocarcinoma (colonic primary), G) Adenocarcinoma (ovarian primary), H) Adenocarcinoma (lung primary), I) reactive mesothelial cells. Viewing Magnification = 600X

Table 1

Analysis of Cadherin Expression for Detection of Malignancy:

E-cadherin Score	No Carcinoma*	Carcinoma
positive	3	28
negative	60	4
Total	63	32

Sensitivity: 87.5%

Specificity: 95%

*Cases of malignant non-epithelial tumors are omitted since they are not expected to express E-cadherin.

Table 2

Analysis of Cytomorphology for Detection of Malignancy:

Cytology Dx	No Tumor	Tumor
positive	0	32
negative	63	7
Total	63	39

Sensitivity: 82%

Specificity: 100%

Table 3

Combined Best Estimate of Final Diagnosis using both Cytomorphology and/or E-cadherin.

Best Combined Diagnosis	No Tumor	Tumor
positive	0	36
negative	63	3*

Sensitivity: 92%*

Specificity: 100%

*These 3 cases included one case of leiomyosarcoma, one case of lymphoma, and a pseudomyxoma peritonei, cases that may not shed sufficient cells into the fluid to make a diagnosis by any method.

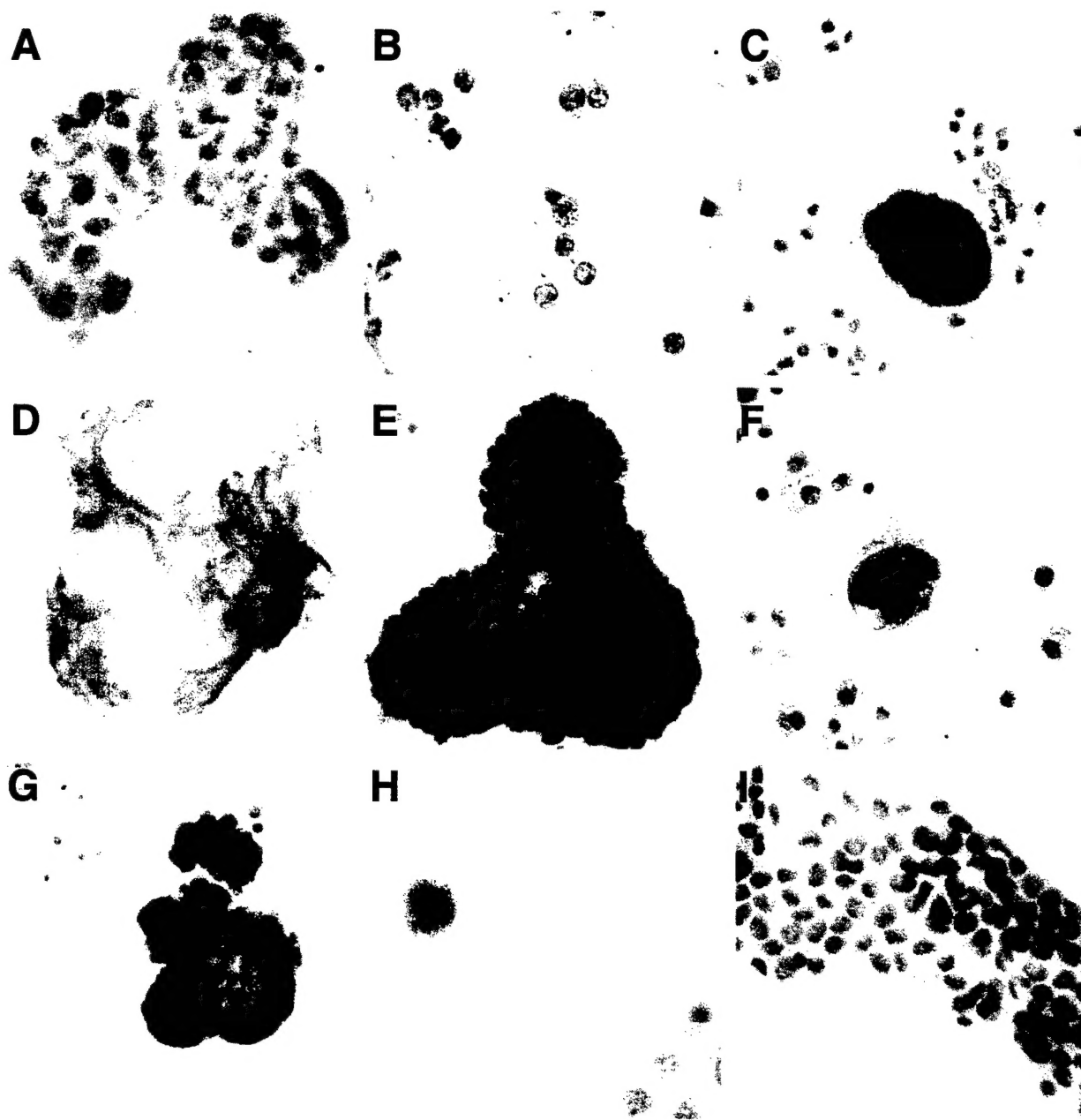


Figure 1
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